ROLE OF ALGINATE LYASE IN THE VIRULENCE OF *PSEUDOMONAS SYRINGAE* PV. *TOMATO* DC3000

By

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DC3000

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CHAPTER I

INTRODUCTION

Pseudomonas syringae is a necrogenic, gram-negative plant pathogen that infects many economically important crops around the world. Strains of *P. syringae* are subdivided into pathovars (pathogenic variants, pvs) based on their host specificity (i. e. *Pseudomonas syringae* pv. *tomato*). *P. syringae* can survive as an epiphyte on leaf surfaces and when the conditions are favorable the bacteria multiply and enter the host plant, which leads to symptom development. During incompatible interactions with resistant host plants, *P. syringae* elicits a plant defense mechanism known as the hypersensitive response (HR). During the HR, plant cells in contact with the pathogen become rapidly necrotic preventing multiplication and spread of the pathogen. The ability of *P. syringae* to cause disease in susceptible host plants or to elicit the hypersensitive response (HR) in resistant plants depends upon the *hrp* (hypersensitive response and pathogenicity) genes as well as a variety of virulence factors.

Among the virulence factors produced by *P. syringae* are phytotoxins (e. g. coronatine), exopolysaccharides (e. g. alginate) and plant growth hormones. In addition, global regulatory genes like *Gac* are also important for pathogenesis. Exopolysaccharides (EPS) are believed to play multiple roles during pathogen-host interactions. For instance, it is assumed that EPS accumulates around the bacterial cells *in planta*, so that moisture is

retained and micro-colonies are protected from sudden desiccation. Furthermore, EPS accumulation might minimize the interaction of the pathogen with the plant cells (Denny, 1995).

P. syringae produces two well characterized exopolysaccharide molecules: (a) levan, a polymer of fructofuranan; and (b) alginate, a polymer of β-D-mannuronate and its C-5 epimer α-L-guluronate, which are arranged in homopolymeric (polymannuronate or polyguluronate) or heteropolymeric block structures (Fett *et al.*, 1986; Gacesa, 1988; Gross & Rudolph, 1987). In addition, bacterial alginates are normally *O*-acetylated (sometimes diacetylated) on the C2 and/or C3 position(s) of the mannuronate residues (Gacesa, 1998). The importance of alginate in the virulence and epiphytic fitness of *P. syringae* has been documented. For example, alginate-deficient mutants of *P. syringae* pv. *syringae* 3525 and FF5 are less virulent and their ability to survive epiphytically is impaired (Penaloza-Vazquez *et al.*, 2004; Yu *et al.*, 1999).

The genes responsible for alginate production have been under scrutiny for the past several years in *P. aeruginosa* and *P. syringae*. It is now accepted that the order and arrangement of the alginate biosynthetic genes in these two pathogens are identical (Buell *et al.*, 2003; Penaloza-Vazquez *et al.*, 1997); however, the environmental signals that trigger the production of alginate are different in these two pseudomonads, which can be explained by the different niches they inhabit. The biosynthetic genes for alginate include *algA*, *C*, *D*, *E*, *F*, *G*, *I*, *J*, *K*, *L*, *X, 8* and *44*. With the exception of *algC*, the biosynthetic genes are clustered together in the genome. Many of the genes on the cluster have been identified as essential for alginate production. Interestingly, the *algL* gene, which encodes alginate lyase was initially shown to be dispensable for alginate production in *P.*

aeruginosa. Similarly in *P. syringae* pv. *syringae*, the *algL* gene was not essential for alginate biosynthesis but was required for optimal alginate production. When the research discussed in this thesis was underway, a paper by Albrecht and Schiller, (Albrecht & Schiller, 2005) demonstrated that *algL* is required for alginate production in *P. aeruginosa* strain FRD1::pJLS3, thereby increasing the controversy surrounding the role of this gene and increasing the impact of studying its function in other pseudomonads like *P. syringae* pv *tomato* (*Pst*) DC3000.

Pst DC3000 is a pathogen of tomato, *Brassica* spp. (cabbage, cauliflower, collard), and *Arabidopsis thaliana* (Moore *et al.*, 1989; Whalen *et al.*, 1991; Zhao *et al.*, 2000). *Pst* DC3000 has gained importance as a model organism for studying plant-microbe interactions, mainly because of its genetic tractability, its pathogenicity on *A. thaliana* and tomato plants, and the availability of its genomic sequence (Buell *et al.*, 2003). In order to better understand the role of alginate lyase in *P. syringae*, an *algL* mutant was constructed in *Pst* DC3000, and the involvement of the *algL* gene in alginate production was investigated. Moreover, the role that *algL* plays during pathogenesis of *Pst* DC3000 on collard plants was also investigated. In summary, this study enhances our understanding of alginate and alginate lyase in plant pathogen interactions.

CHAPTER II

REVIEW OF LITERATURE

Pseudomonas syringae

Pseudomonas syringae is a necrogenic, gram-negative phytopathogenic bacterium. In general, *P.* sy*ringae* strains spend part of their life cycle residing as epiphytes on host or non-host plants. When conditions are favorable, *P. syringae* enters susceptible host plants through wounds, stomata and other natural openings. Once inside the plant, the bacteria colonize the intercellular space (the apoplast) and disseminate to other parts of the plant (Preston, 2000). The ability of *P. syringae* to multiply and kill plant cells depends on various pathogenicity and virulence factors (Alfano & Collmer, 1996). One of the most important pathogenicity factors is the type III secretion system (TTSS), which is encoded by the *hrp* (hypersensitive response and pathogenicity) genes (He, 1998; He *et al.*, 2004; Jin *et al.*, 2003). *P. syringae* presumably uses the TTSS to deliver effector proteins inside the plant cell, and these proteins govern the outcome of the interaction (e.g. compatible or incompatible) (Alfano & Collmer, 1996; Preston, 2000).

In addition to the TTSS, *P. syringae* strains produce virulence factors that enhance the severity of the disease during pathogen-host interactions. Among these virulence factors are (a) phytotoxins (e.g coronatine) (Bender *et al.*, 1999), (b)

exopolysaccharides (e.g. alginate) (Penaloza-Vazquez *et al.*, 2004; Yu *et al.*, 1999), and (c) plant growth hormones and plant cell wall degrading enzymes (Alfano & Collmer, 1996; Buell *et al.*, 2003).

Based on their host specificity, strains of *P. syringae* are subdivided into pathovars (pathogenic variants, pvs.), which cause a wide variety of diseases including wildfire of tobacco (*P. syringae* pv. *tabacci*), angular leaf spot of cucumber (*P. syringae* pv. *lacrymans*), halo blight of beans (*P. syringae* pv. *phaseolicola*), citrus blast, pear blast, bean leaf spot, and lilac blight (*P. syringae* pv. *syringae*), bacterial blight of soybean (*P. syringae* pv. *glycinea*), leaf spot of *Brassica* spp (*P. syringae* pv. *maculicula*) and bacterial speck of tomato (*P. syringae* pv. *tomato*) (Agrios, 1997). In non-host or resistant plants, *P. syringae* elicits a plant defense mechanism known as hypersensitive response (HR). The HR is a complex form of a programmed plant cell death that is generally characterized by brown dead cells at the site of infection that prevents the spread of the pathogen (Heath, 2000).

***Pseudomonas syringae* pv. *tomato* DC3000**

Pseudomonas syringae pv. *tomato* (*Pst*) DC3000 is a pathogen of tomato, *Brassica* spp. (cabbage, cauliflower, collard), and *Arabidopsis thaliana* (Moore *et al.*, 1989; Whalen *et al.*, 1991; Zhao *et al.*, 2000). *Pst* DC3000 has gained importance as a model organism for studying plant-microbe interactions, mainly because of its genetic tractability, its pathogenicity on *A. thaliana* and tomato plants, and the availability of its genomic sequence (http://pseudomonas-syringae.org/pst_DC3000_gen.htm) (Buell *et al.*, 2003). In tomato plants, *Pst* DC3000 causes necrotic leaf spots that are often surrounded by

chlorotic margins (Preston, 2000). In collard plants, *Pst* DC3000 induces water-soaked, spreading lesions that sometimes are surrounded by chlorotic margins (Keith *et al.*, 2003).

The genome of *Pst* DC3000 consists of a circular chromosome (6,397,126 bp) and two plasmids (73,661 bp and 67,473 bp). A total of 5,763 open reading frames (ORFs) have been identified within the genome. Among the predicted genes, 298 were identified as virulence genes (Buell *et al.*, 2003). In *Pst* DC3000-host interactions, the TTSS and the phytotoxin coronatine are important for symptoms, disease development, and multiplication of the bacterium *in planta* (Brooks *et al.*, 2004; Jin *et al.*, 2003). In addition, alginate, a copolymer of β-D-mannuronate and its C-5 epimer α-L-guluronate (Fett, 2001), has been implicated as a virulence factor in some *Pseudomonas* spp., including the phytopathogen *P. syringae* pv. *syringae* and the human opportunist pathogen *P. aeruginosa* (Penaloza-Vazquez *et al.*, 2004; Ramsey & Wozniak, 2005; Yu *et al.*, 1999). In the case of *Pst* DC3000, the alginate genes are expressed within the water-soaked lesions of collard plants; suggesting that alginate is important for the pathogenesis of *Pst* DC3000 (Keith *et al.*, 2003).

The hypersensitive response and the *hrp* cluster

The hypersensitive response (HR) is an important defense reaction in plants that generally occurs during incompatible interactions of host plants and phytopathogens (Heath, 2000). During the HR, physiological changes occur within plant cells at the infection site. These changes include loss of permeability in cell membranes, production of reactive oxygen species, increased respiration, accumulation and oxidation of phenolic

compounds, and production of phytoalexins (He, 1998). The outcome of these physiological changes is collapse and death of the cells at or near the site of infection. In susceptible varieties, such changes either do not occur or occur more slowly.

The elicitation of the HR depends upon the *hrp* genes (He, 1998) and the *hrp*-encoded TTSS, which is present in many animal and plant pathogenic bacteria (He, 1998). Some of the *hrp* genes are highly conserved among bacteria and are hence called the *hrc* genes (for *h*ypersensitive *r*esponse and *c*onserved) (Bogdanove *et al.*, 1996). The *hrp/hrc* regulon of some *P. syringae* strains consists of 27 ORFs, which can be subdivided into three different classes of genes. The first class contains genes that encode the core components of the TTSS, a second class encodes regulatory proteins, and the third class contains genes that encode the effector proteins that are secreted via the TTSS (Alfano *et al.*, 2000). During compatible interactions, the TTSS presumably delivers effector proteins inside the host cells (Jin *et al.*, 2003). These effector proteins suppress the activation of plant defense response, which ultimately leads to bacterial multiplication and disease (Jin *et al.*, 2003).

The phytotoxin coronatine

In addition to effector proteins secreted via the TTSS, many pathovars of *P. syringae* (including *Pst* DC3000) produce the phytotoxin coronatine (Bender *et al.*, 1999). Coronatine is a non-host-specific phytotoxin that consists of two distinct moieties, the polyketide coronafacic acid (CFA) and coronamic acid (CMA), which is an ethylcyclopropyl amino acid (Ichihara *et al.*, 1977; Parry *et al.*, 1994). Coronatine production *in planta* can elicit a wide array of effects including chlorosis, hypertrophy,

inhibition of root elongation and the stimulation of ethylene production (Bender *et al.*, 1999; Kenyon & Turner, 1992). Coronatine has been documented as a virulence factor in multiple *P. syringae* pathovars including pvs. *glycinea*, *tomato* and *maculicola.* Coronatine-defective mutants of these pathovars are less virulent compared with the wild-type parental strains (Bender *et al.*, 1987; Budde & Ullrich, 2000; Tamura *et al.*, 1998). In *Pst* DC3000, coronatine-deficient mutants cause reduced symptoms on tomato and *A. thaliana*, and the bacterial population *in planta* is reduced when compared with the wild-type *Pst* DC3000 (Brooks *et al.*, 2004; Mittal & Davis, 1995).

Exopolysaccharides produced by *P. syringae*

P. syringae is known to produce two different exopolysaccharide (EPS) molecules: (a) levan, a polymer of fructofuranan; and (b) alginate (Fett *et al.*, 1986; Gross & Rudolph, 1987). Bacterial alginates are composed of β-D-mannuronate and its C-5 epimer α-L-guluronate (Gacesa, 1988) arranged in homopolymeric (polymannuronate or polyguluronate) or heteropolymeric block structures (**Figure 1**). In addition, bacterial alginates are normally *O*-acetylated (sometimes diacetylated) on the C2 and/or C3 position(s) of the mannuronate residues (Gacesa, 1998).

The importance of alginate as a virulence factor has been primarily investigated in the human opportunistic pathogen, *Pseudomonas aeruginosa* (McAvoy *et al.*, 1989). *P aeruginosa* produces alginate during chronic lung infections in patients with cystic fibrosis where it presumably functions as a physical barrier that restricts the diffusion of antibiotics and protects the bacterial cells from phagocytosis (May *et al.*, 1991). Alginate is also produced by other *Pseudomonas* spp. including *P. cichorii*, *P. corrugata*, *P.*

fluorescens, *P. mendocina*, *P. putida*, *P. syringae* and *P. viridiflava* (Fett, 2001). Furthermore, bacteria in the genus *Azotobacter* (Clementi, 1997) and the marine algae *Phaeophyceae* (Evans, 1989) also synthesize alginate. An important difference in the structure of these alginates is that *Pseudomonas* spp. produce acetylated alginates whereas algal and *Azotobacter* alginates lack acetyl groups.

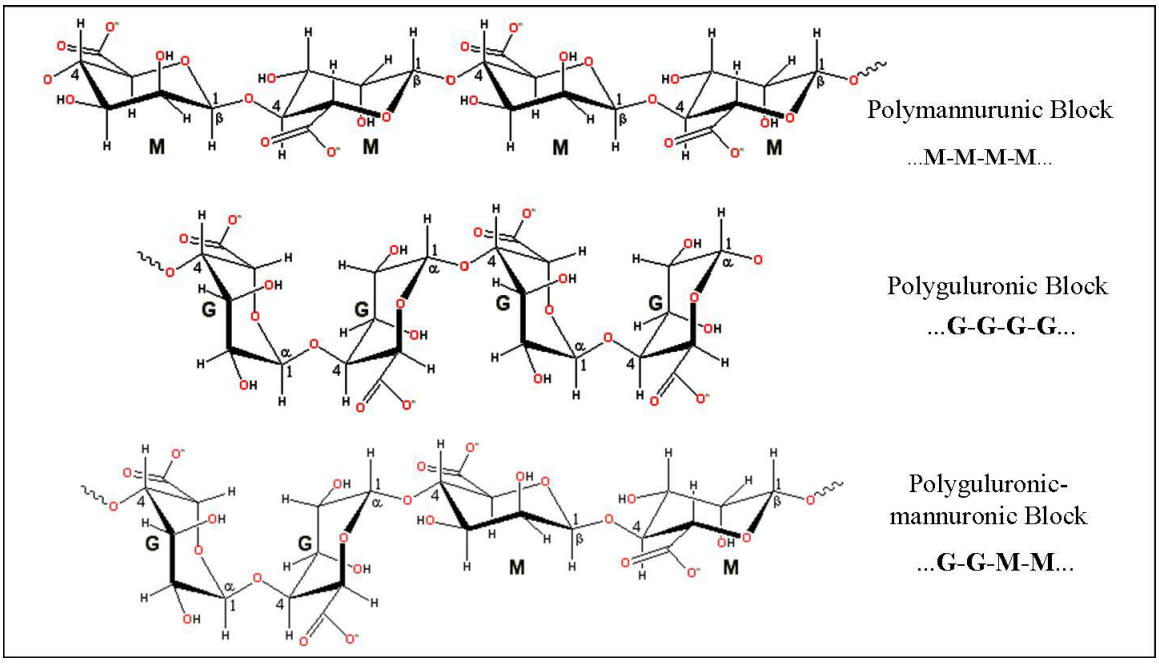

Figure 1: Homopolymeric and heteropolymeric structure of alginates. Algal and *Azotobacter* spp. alginates consist of polymannuronic and/or polyguluronic structures. In *P. aeruginosa* and *P. syringae*, the polyguluronic blocks structures are not present. Furthermore, mannuronic residues are acetylated at C2 or C3 position (not shown) (Gacesa, 1998).

Role of alginate in *P. syringae*

Exopolysaccharides have multiple functions in pathogen-host interactions. The main hindrance to bacterial multiplication in the intercellular space of plants is the scarcity of water (Rudolph & Bernd, 1997). It is assumed that EPS accumulates around the bacterial cells *in planta*, so that moisture is retained and micro-colonies are protected from sudden desiccation (Denny, 1995). Moreover, during pathogenesis EPS might

benefit the pathogen by reducing water evaporation in host tissues, minimizing interaction with plant cells and promoting colonization (Denny, 1995).

In plants, early experiments suggested that purified EPS (including alginate) from different pseudomonads and xanthomonads might induce water-soaked lesions in host plants (El-Banoby & Rudolph, 1979); however, the involvement of EPS in the formation of water-soaked lesions was not conclusive. Later, it was shown that alginate is the major EPS produced in water-soaked lesions of leaves inoculated with different *P. syringae* pathovars (Fett & Dunn, 1989). This study suggested, but did not prove, that alginate was responsible for the water-soaked appearance of lesions.

Studies investigating the role of alginate in the virulence of *P. syringae* pv. *syringae* 3525, which causes bacterial brown spot on bean, were performed by Yu et al. (Yu *et al.*, 1999). An alginate-defective mutant derived from strain 3525 was impaired in its ability to colonize the surface of tomato leaves (a non-host), indicating that alginate contributes to the epiphytic fitness of strain 3525. Furthermore, the mutant produced fewer symptoms on beans leaves (the host), and the bacterial population of the mutant was significantly lower than the wild-type. Collectively these results indicated that alginate contributes to virulence and epiphytic fitness of *P. syringae* pv. *syringae* 3525 (Yu *et al.*, 1999).

More recently, gene expression studies indicated that alginate biosynthetic genes are expressed during the development of water-soaked lesions in *P. syringae*-host interactions (Keith *et al.*, 2003). The expression of *algD*, which encodes a key enzyme in the alginate biosynthetic pathway, correlated with the development of water-soaked lesions in collard leaves. This study provided additional evidence that alginate genes are

expressed within water-soaked lesions during infection (Keith *et al.*, 2003). However, genetic proof that alginate was responsible for the water-soaked lesions was not provided in this study (Keith *et al.*, 2003).

Environmental signals that stimulate alginate production in *P. syringae*

In *Pseudomonas* spp., the alginate biosynthetic genes are normally silent (e.g. not expressed) *in vitro*. However, some *Pseudomonas* spp. showing a nonmucoid phenotype *in vitro* can synthesize alginate *in planta* (e.g. *P. syringae*) or in the lungs of patients with cystic fibrosis (e.g. *P. aeruginosa*). The switch from a nonmucoid to a mucoid phenotype depends on the presence of environmental factors that activate the expression of alginate regulatory genes, which ultimately leads to alginate production.

Much of the information regarding the activation of alginate genes is from studies conducted with *P. aeruginosa*. Some of the factors known to induce alginate production in *P. aeruginosa* also activate alginate synthesis in *P. syringae*, including exposure to oxidative stress, high osmotic conditions, and dehydration. However, some of the environmental signals for alginate gene expression in *P. syringae* differ from *P. aeruginosa* (Penaloza-Vazquez *et al.*, 1997). For example, copper ions can trigger alginate production and alginate gene expression in *P. syringae*, but copper is not a signal for alginate synthesis in *P. aeruginosa* (Kidambi *et al.*, 1995). The exposure of bacterial cells to Cu^{2+} ions can generate free radicals, which leads to oxidative stress (Keith & Bender, 1999). Accordingly, copper increased alginate production in certain strains of *P. syringae* (Kidambi *et al.*, 1995). Copper also activates transcription of *algD* (Penaloza-Vazquez *et al.*, 1997), which encodes GDP-mannose dehydrogenase (Deretic *et al.*,

1987). *algD* is the first gene to be transcribed in the alginate gene clusters of *P. aeruginosa* and *P. syringae*, and transcription from the *algD* promoter region is tightly controlled in both pathogens (Penaloza-Vazquez *et al.*, 1997; Ramsey & Wozniak, 2005). In *P. syringae*, exposure to copper also activates the expression of *algT*, which encodes the alternative sigma factor σ^{22}, required for activation of *algD* transcription (Keith & Bender, 1999).

During colonization of plant tissue, *P. syringae* may be exposed to hydrogen peroxide (H_2O_2), another source of oxidative stress, which also increases the transcription of *algT in vitro* (Keith & Bender, 1999). Expression of *algD* was also observed during the HR in tobacco plants infected with *Pst* DC3000 (Keith *et al.*, 2003). The HR is often associated with an oxidative burst, which involves the production of potentially cytotoxic quantities of H_2O_2 and $O_2^{\cdot-}$ (Keith *et al.*, 2003; Wojtaszek, 1997).

Elevated osmotic conditions (e.g. NaCl, 0.2-0.7 M) and dehydration (ethanol, 3-5%) enhanced alginate production in pathogenic and nonpathogenic fluorescent pseudomonads. De Vault et. al. (De Vault *et al.*, 1990) reported that the addition of 1% ethanol to broth media caused a two-fold increase in alginate production by mucoid *P. aeruginosa* strain 8821, but did not enhance the synthesis of alginate in the nonmucoid strains PA01 and 8822 (the latter strain is a spontaneous, nonmucoid derivative of 8821) (De Vault *et al.*, 1990). Singh et. al. (Singh *et al.*, 1992) showed increased alginate production (up to 22-fold higher levels) in *P. syringae* pv. *glycinea*, *P. viridiflava* and *P. fluorescens* by the addition of 0.2-0.5 M NaCl. However, at levels above 0.5 M NaCl, growth of the bacteria was reduced. The addition of sorbitol (a nonionic compound that increases osmotic environment) gave similar results without reducing growth (Singh *et*

al., 1992). NaCl also enhanced *algD* expression in both *P. syringae* and *P. aeruginosa* (Berry *et al.*, 1989). Collectively, these results indicate that dehydration and osmotic stress are conserved signals for alginate gene activation and alginate production.

Genes involved in the biosynthesis and regulation of alginate in *Pseudomonas* spp.

The genes responsible for alginate biosynthesis and regulation have been identified in *Pseudomonas* and *Azotobacter* spp. The genes are arranged in three clusters throughout the genome (**Figure 2**). The majority of the genes involved in alginate biosynthesis are grouped in one cluster, whereas the other two clusters are involved in the regulation of alginate biosynthesis and in the switch from the nonmucoid to mucoid mode of growth.

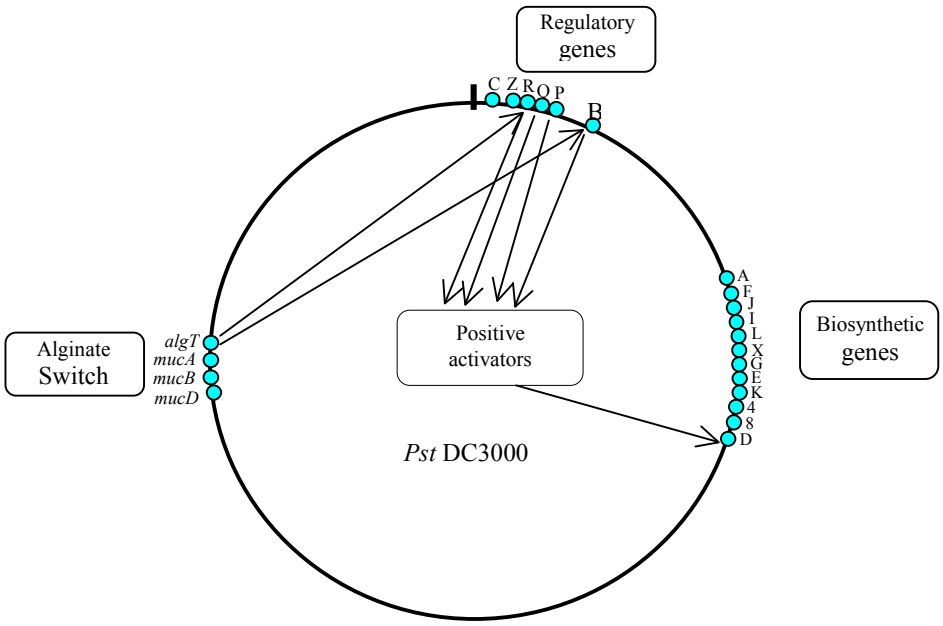

Figure 2: Physical map of alginate genes in *Pst* DC3000.

The genes involved in alginate biosynthesis include *algA*, *C*, *D*, *E*, *F*, *G*, *I*, *J*, *K*, *L*, *X, 8* and *44* (**Table 1**). With the exception of *algC*, the biosynthetic genes are clustered

together (**Figure 2**). The order and arrangement of the biosynthetic genes in *P. syringae* is identical to that in *P. aeruginosa* (Buell *et al.*, 2003; Penaloza-Vazquez *et al.*, 1997). The cluster of genes responsible for the conversion to the mucoid mode of growth includes *algT* (*algU*), *mucA*, *mucB*, and *mucD* (**Table 1**). The regulatory cluster includes the genes *algB*, *algR3*(*algP*), *algR*(*algR1*), *algQ*(*algR2*), and *algZ* (**Table 1**), which have a positive regulatory effect on transcription of the *algD* promoter. In contrast, *mucA*, *mucB* and *mucD*, negatively impact the *algD* promoter by inhibiting the activity of the *algT* product, σ^{22}.

Table 1: Genes involved in alginate biosynthesis and regulation in *Pst* DC3000.

Gene	Additional Name(s)	Location in *Pst* DC3000 (bp)	Function	References
			Alginate Biosynthesis	
algA		1351082…1352533	Phosphomannose isomerase and GDP-mannose pyrophosphorylase activities	(Shinabarger *et al.*, 1991)
algC		103735…105132	Phosphomannose and phosphoglucomutase activities; also involved in LPS biosynthesis	(Coyne *et al.*, 1994; Regni *et al.*, 2004; Zielinski *et al.*, 1991)
algD		1366206…1367522	GDP-mannose dehydrogenase	(Koopmann *et al.*, 2001; Snook *et al.*, 2003)
algE		1360516…1361997	Outer membrane, alginate-specific ion channel responsible for alginate export	(Chu *et al.*, 1991; Grabert *et al.*, 1990)
algF		1352670…1353338	Periplasmic protein involved in alginate *O*-acetylation	(Franklin & Ohman, 2002)
algG		1358879…1360489	Periplasmic-associated, C5-mannuronan epimerase; protects alginate polymer from lyase activity	(Gimmestad *et al.*, 2003; Jain *et al.*, 2003)
algI		1354537…1356093	Inner membrane protein involved in alginate *O*-acetylation	(Franklin & Ohman, 2002)
algJ		1353351…1354526	Membrane-associated, periplasmic protein involved in alginate *O*-acetylation	(Franklin & Ohman, 2002)
algK		1361994…1363406	Periplasmic protein required for proper polymer formation	(Aarons *et al.*, 1997; Sumita & Ohman, 1998)

Gene	Additional Name(s)	Location in *Pst* DC3000 (bp)	Function	References
algL		1356280…1357416	Periplasmic alginate lyase	(Monday & Schiller, 1996; Preston *et al.*, 2000)
algX		1357425…1358864	Periplasmic protein required for proper polymer formation	(Monday & Schiller, 1996; Robles-Price *et al.*, 2004)
alg8	PSPT01241	1364622…1366103	Membrane protein: hypothesized polymerase	(Maharaj *et al.*, 1993; Mejia-Ruiz *et al.*, 1997)
alg44	PSPT01242	1363409…1364581	Membrane protein required for alginate production	(Maharaj *et al.*, 1993; Mejia-Ruiz *et al.*, 1997)
			Alginate Regulation	
algB		363589…364935	Positive activator for *algD* transcription	(Goldberg & Dahnke, 1992; Wozniak & Ohman, 1991)
algR3	*algP,* PSPT00136	159053…160009	Histone-like protein; activates *algD* transcription	(Kato *et al.*, 1990; Konyecsni & Deretic, 1990)
algR	*algR1*	151591…152337	Positively activates *algC* and *algD* transcription	(Deretic & Konyecsni, 1989; Penaloza-Vazquez *et al.*, 2004)
algT	*algU*, *rpoE*	4757624…4758205	Alternative sigma factor responsible for transcription of *algD*, *algR*, *algT* and *algZ*	(Hershberger *et al.*, 1995; Keith & Bender, 1999; Wozniak *et al.*, 2003)
algQ	*algR2*	157604…158077	Positive activator of *algD* transcription	(Deretic & Konyecsni, 1989; Kato *et al.*, 1989; Ledgham *et al.*, 2003)
algZ	PSPT00126	150512…151594	Activator of *algD* transcription	(Baynham *et al.*, 1999)
mucA		4757000…4757590	Inner membrane protein; anti-sigma factor that negatively regulates alginate production by sequestration of *algT*	(Keith & Bender, 2001; Martin *et al.*, 1993; Mathee *et al.*, 1997)
mucB		4756035…4756991	Periplasmic protein that negatively regulates *algT*; inactivation leads to alginate production	(Goldberg *et al.*, 1993; Keith & Bender, 2001; Martin *et al.*, 1993)
mucD	PSPT04221	4754310…4755749	Negatively regulates *algT* by removing activating factors	(Boucher *et al.*, 1996; Yorgey *et al.*, 2001)

The alginate biosynthetic pathway in *Pseudomonas* spp. is shown in **Figure 3** (Govan & Deretic, 1996). Biosynthesis begins with fructose 6-phosphate, which is converted into mannose 6-phosphate via phosphomannose isomerase (PMI; encoded by *algA*). Mannose 6-phosphate is then converted to mannose 1-phosphate by phosphomannomutase (PMM; encoded by *algC*). Conversion of mannose 1-phosphate to GDP-mannose is catalyzed by GDP-mannose pyrophosphorylase (GMP; also encoded by *algA*). The conversion of GDP-mannose into the GDP-mannuronate monomer via GDP-mannose dehydrogenase (GMD; encoded by *algD*) is a rate-limiting step in the biosynthesis of alginate. GDP-mannuronate residues are then polymerized, epimerized and acetylated to form mature alginate (**Figure 3**) (Ramsey & Wozniak, 2005). Jain and Ohman (Jain & Ohman, 1998) demonstrated that an *algK* mutant secreted monomeric uronic acids, providing evidence for the role of AlgK in the polymerization of alginate. AlgX is also required for alginate synthesis and may have a role in polymerization (Monday & Schiller, 1996), thus facilitating the transfer of an acetyl group from acetyl-CoA to a transacetylase. This transacetylase is the product of gene *algF*. Two other genes, *algI* and *algJ* (Franklin & Ohman, 1993; Franklin & Ohman, 1996) are required for the acetylation of alginate. Another alginate modifying enzyme, AlgG, catalyzes the epimerization of the mannuronate residues at the C-5 position, thus introducing guluronate residues into polymannuronate (Jain *et al.*, 2003). In addition to acetylation and epimerization, a major modification of *Pseudomonas* spp. alginate is catalyzed by alginate lyase, which is encoded by *algL*. AlgL physically degrades alginate and may have a role in detachment of bacterial cells (Monday & Schiller, 1996). Transport of

alginate to the extracellular milieu may occur via an outer membrane porin encoded by *algE* (Rehm *et al.*, 1994).

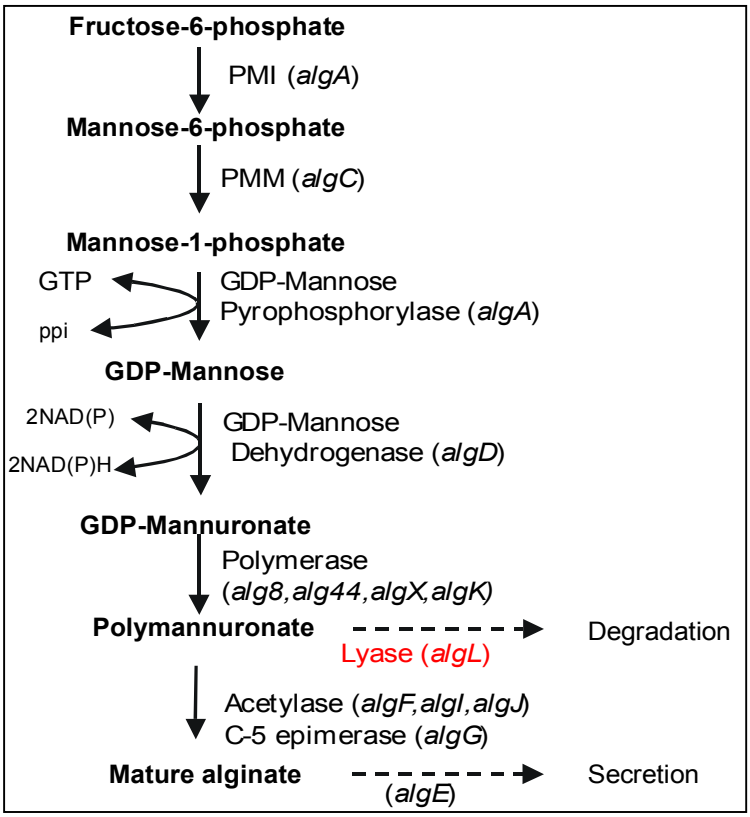

Figure 3: The biosynthesis of alginate by *Pseudomonas spp.* Fructose-6-phosphate is converted to GDP-mannuronate, thus providing mannuronate residues for polymerization. Guluronate residues may be incorporated into the polymer via the epimerization of mannuronate, a reaction catalyzed by AlgG. Mannuronic residues of bacterial alginates are partially *O*-acetylated by the gene products of *algF*, *algI* and *algJ*. Secretion of mature alginate is presumably catalyzed by the AlgE protein.

Alginate lyase

Alginate-degrading enzymes are produced by a variety of organisms; including marine algae, marine mollusks and a broad range of microorganisms (Wong *et al.*, 2000). The alginate-degrading enzyme, alginate lyase, is produced by many organisms that do not synthesize alginate including marine bacteria, soil bacteria and fungi (Wong *et al.*,

2000). In many of these microorganisms the production of alginate lyase is associated with the utilization of alginate as carbon and energy source (Wong *et al.*, 2000). However, there are several bacteria in the genera *Pseudomonas* and *Azotobacter* that produce both alginate and alginate lyase (AlgL).

AlgL in *P. aeruginosa*, *P. syringae* and *A. vinelandii* is encoded by the *algL* gene, which is located within the alginate biosynthetic cluster (Ertesvag *et al.*, 1998; Preston *et al.*, 2000; Schiller *et al.*, 1993). The *algL* gene is also present in *P. syringae* pv. *syringae* (Penaloza-Vazquez *et al.*, 1997; Yu *et al.*, 1999) and *P. syringae* pv. *tomato* DC3000 (Buell *et al.*, 2003). Among *Pseudomonas* spp., alginate lyase has been biochemically characterized from *P. aeruginosa* (Schiller *et al.*, 1993), *P. alginovora* (Chavagnat *et al.*, 1996) and *P. syringae* (Ott *et al.*, 2001; Preston *et al.*, 2000). In *Azotobacter*, the biochemical properties of AlgL have been studied in *A. vinelandii* (Ertesvag *et al.*, 1998) and *A. chroococcum* (Haraguchi & Kodama, 1996).

Alginate lyase in *Pst* DC3000

The *algL* gene in *Pst* DC3000 is located within the alginate biosynthetic cluster (**Figure 2**). The arrangement of the cluster is identical to other alginate-producing bacteria. The size of *algL* in *Pst* DC3000 is similar to *algL* in other *Pseudomonas* and *Azotobacter spp*. In addition, the putative amino acid sequence of AlgL shows similarity to other known alginate lyases (**Table 2**).

Table 2: Size of *algL* and relatedness of alginate lyase in *Pst* DC3000 to AlgL in other alginate-producing bacteria.

Strain	Gene size (bp)	% amino acid similarity of *Pst* DC3000 alginate lyase
Pst DC3000	1137	100
P. s. pv. syringae B728a	1137	99
P. s. pv. syringae FF5	1137	99
P. aeruginosa	1104	74
A. vinelandii	1125	75
A. chroococcum	1302	75

Role of alginate lyase in alginate-producing bacteria

AlgL (EC4.2.2.3) is a depolymerizing enzyme synthesized in both alginate-producing and alginate-nonproducing bacteria (Wong *et al.*, 2000). Depolymerization of alginate by AlgL is thought to occur via β-elimination by cleaving the 1-4 glycosidic linkage of the polymer (**Figure 4**), resulting in an unsaturated non-reducing terminus (Gacesa, 1992; Haugen *et al.*, 1990).

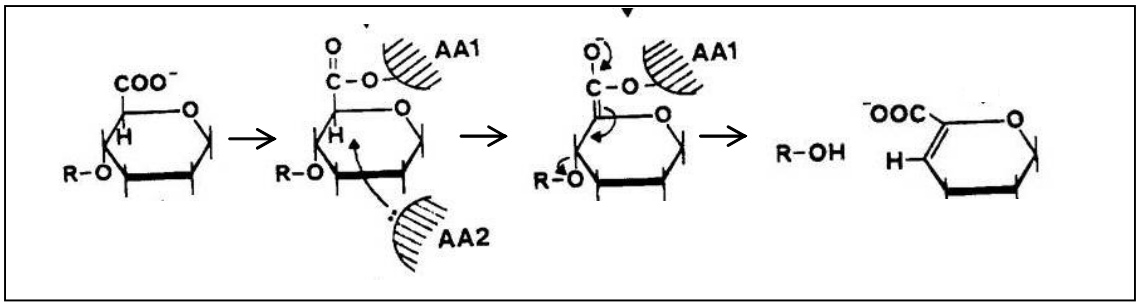

Figure 4: A proposed catalytic mechanism for alginate lyase. AA1 and AA2 represent amino acid residues within the active site of the enzyme (Gacesa, 1992).

The role of AlgL in alginate-nonproducing bacteria is to provide alginate monomers as a carbon source (Wong *et al.*, 2000). However, in alginate-producing bacteria, the role of AlgL remains uncertain. In *P. aeruginosa*, *P. syringae* and *A. vinelandii*, AlgL is thought to be involved in alginate synthesis, alginate modification and

in the mucoid colony morphology (Albrecht & Schiller, 2005; Boyd & Chakrabarty, 1994; Penaloza-Vazquez *et al.*, 1997; Yu *et al.*, 1999).

Schiller et al. (Schiller *et al.*, 1993) reported the cloning and sequencing of *algL* from *P. aeruginosa*. The *algL* gene in *P. aeruginosa* is activated by environmental signals (e.g. elevated osmolarity) that also induce the alginate biosynthetic genes (Berry *et al.*, 1989; Schiller *et al.*, 1993). These observations suggest that AlgL in *P. aeruginosa* is produced along with the synthesis of alginate. In mucoid *P. aeruginosa*, production of alginate is linked to early steps during infection by facilitating the attachment of the bacteria to different surfaces. Moreover, attachment to surfaces seems to stimulate alginate production (Boyd & Chakrabarty, 1994). Overproduction of AlgL in mucoid *P. aeruginosa* increased the degradation of alginate and hence reduced polymer length, which ultimately increased detachment of bacteria from surfaces. According to Boyd and Chakrabarty (Boyd & Chakrabarty, 1994) these results suggest a possible role for AlgL in degrading alginate and hence facilitating dissemination of the bacteria to a more suitable habitat. Another possible role for AlgL was suggested by May and Chakrabarty (May & Chakrabarty, 1994), who proposed that AlgL may be an editing protein that controls alginate polymer length or an enzyme that provides short oligomers that may initiate the process of polymerization.

In the alginate-producer *A. vinelandii*, production of AlgL results in a low molecular weight alginate polymer, and construction of a non-polar *algL* mutant of *A. vinelandii* resulted in a mutant strain that produced alginate of high molecular weight (Trujillo-Roldan *et al.*, 2003). This result indicates that AlgL degrades alginate during biosynthesis and that *algL* is expressed altogether with the alginate biosynthetic genes but

is not required for alginate synthesis and secretion in *A. vinelandii* (Trujillo-Roldan *et al.*, 2003).

In *P. syringae,* AlgL is involved in the modification of nascent alginate. In *P. syringae* pv. *phaseolicola,* Ott et al. (Ott *et al.*, 2001) demonstrated that AlgL is primarily secreted to the extracellular milieu during mid-exponential and late-stationary phases of growth. In contrast, intracellular AlgL activity was low, suggesting that AlgL may have an extracellular function, perhaps by modifying extracellular alginate.

Although some progress has been made in elucidating the role of alginate lyase in *P. aeruginosa*, *P. syringae* and *A. vinelandii*, there is still controversy on the absolute requirement of AlgL in synthesis of alginate. For example, Boyd and Chakrabarty (Boyd & Chakrabarty, 1994), reported that alginate lyase was not required for alginate synthesis in *P. aeruginosa.* However, a recent paper by Albrecht and Schiller, (Albrecht & Schiller, 2005), increased the controversy about the requirement of AlgL during alginate biosynthesis in *P. aeruginosa*. They constructed a non-polar mutation in the *algL* gene of *P. aeruginosa* strain FRD1::pJLS3. The *algL* mutant was unable to produce alginate unless the *algL* gene was expressed *in trans*. Moreover they created site-directed mutations in the *algL* gene that abolished the activity of the enzyme. None of the mutant forms of *algL* complemented the *algL* non-polar mutant; thus they concluded that a functional AlgL is required for alginate production. Finally, they speculate that AlgL is part of the scaffold complex along with AlgG, AlgX, and AlgK, and formation of this scaffold is required for alginate production.

In order to better understand the role of alginate lyase in *P. syringae*, an *algL* mutant of *P. syringae* pv. *tomato* DC3000 was constructed by Dr. Chris Allen, a former

postdoctoral researcher in the Bender laboratory. The *algL* mutant of *Pst* DC3000 (named CA1046) was generated by disrupting the chromosomally-encoded *algL* gene by inserting a kanamycin resistance cassette. The mutation in *Pst* DC3000 was confirmed by Southern blot analysis, and preliminary data showed that CA1046 was impaired in its ability to cause disease in collard plants (Allen and Bender, unpublished). However, the *algL* mutant of *Pst* DC3000 was not phenotypically characterized, genetically complemented or analyzed for pathogenicity, which are objectives of this study.

CHAPTER III

ROLE OF *ALGL* (ALGINATE LYASE) OF *P. SYRINGAE* PV. *TOMATO* DC3000 IN THE BIOSYNTHESIS OF ALGINATE AND VIRULENCE ON COLLARD PLANTS

ABSTRACT

Pseudomonas syringae pv. *tomato* (*Pst*) DC3000 produces the exopolysaccharide alginate, a copolymer of *O*-acetylated β-1,4 linked D-mannuronic and L-guluronic acid. Alginate has been reported to be important during pathogenesis in *P. syringae* and *P. aeruginosa*. The alginate biosynthetic genes in *Pst* DC3000 include *algD-8-44-K-E-G-X-L-I-J-F-A* and *algC*. With the exception of *algC*, all the genes are clustered together and controlled by the *algD* promoter region. The *algL* gene encodes alginate lyase, an enzyme capable of degrading alginate. The role of *algL* in alginate biosynthesis and pathogenicity has not been evaluated previously in *P. syringae*. In this study, a non-polar *algL* mutation of *Pst* DC3000 was generated. The *algL* mutant, named CA1046, was able to produce alginate *in vitro* suggesting that *algL* may not be required for alginate production *in vitro*. When analyzed for pathogenicity in collard plants, CA1046 was greatly impaired in its ability to cause symptoms in collard leaves. The total and internal population dynamics further demonstrated that CA1046 is capable of entering collard leaves but incapable of surviving and multiplying inside this host. Moreover, infiltration

of collard leaves with CA1046 carrying a GFP expression vector demonstrated that CA1046 is not able to survive inside collard leaves three days after infiltration. These results correlate with the population dynamics. Survival of CA1046 was also evaluated in tomato (host) and tobacco (non-host) leaves. The results showed that CA1046 is able to survive on tomato and tobacco, suggesting that the *algL* mutation did not affect survival of CA1046 in these two plants. These results correlate with a previous study where the *algD* gene, which represents the committed step for alginate biosynthesis, was highly expressed in collard leaves whereas *algD* expression was lower in tomato and tobacco. In the present study, complementation studies demonstrated that pathogenicity of CA1046 can be restored with the whole alginate cluster, a part of the cluster (*algX-algA*) or the *algL* gene alone. In summary, this study shows that the *algL* gene in *Pst* DC3000 is extremely important for pathogenicity in collard plants but not in tomato. Furthermore, the results of this study indicate that the *algL* gene is not required for *in vitro* production of alginate by *Pst* DC3000.

INTRODUCTION

Exopolysaccharides (EPS) molecules are carbohydrate polymers produced by many pathogenic and non-pathogenic bacteria (Leigh & Coplin, 1992). Bacterial EPS can form a loosely associated extracellular slime or remain closely attached to cells as a capsule layer; moreover, in some instances, they can be secreted to the growth media (Whitfield, 1988). The production of EPS presumably confers a selective advantage to plant pathogenic bacteria by protecting them from the stressful conditions that occur

during plant-host interactions. It is assumed that during pathogen-host interactions, EPS molecules accumulate around the bacterial cells *in planta*, so that moisture is retained and micro-colonies are protected from sudden desiccation (Denny, 1995). Moreover, during pathogenesis, EPS might benefit the pathogen by reducing water evaporation in host tissues, thereby minimizing interaction with plant cells and promoting colonization (Denny, 1995).

P. syringae produces two well-characterized EPS molecules: (a) levan, a polymer of fructofuranan; and (b) alginate, a polymer of β-D-mannuronate and its C-5 epimer α-L-guluronate (Fett *et al.*, 1986; Gacesa, 1988; Gross & Rudolph, 1987). Several other pseudomonads, including the human opportunistic pathogen *P. aeruginosa,* produce alginate (McAvoy *et al.*, 1989). *P. aeruginosa* produces alginate during chronic lung infections in patients with cystic fibrosis where it presumably functions as a physical barrier that restricts the diffusion of antibiotics and protects the bacterial cells from phagocytosis (May *et al.*, 1991). Furthermore, bacteria in the genus *Azotobacter* (Clementi, 1997) and the marine algae *Phaeophyceae* (Evans, 1989) also synthesize alginate. An important difference in the structure of these alginates is that *Pseudomonas* spp. produce alginates that are normally *O*-acetylated (sometimes diacetylated) on the C2 and/or C3 position(s) of the mannuronate residues (Gacesa, 1998), whereas algal and *Azotobacter* alginates lack acetyl groups.

The virulence of *P. syringae* and its capacity to survive as an epiphyte has been correlated with its ability to produce alginate *in planta* (Fett & Wijey, 1995; Yu *et al.*, 1999). Additionally, gene expression studies indicated that alginate biosynthetic genes are expressed during the development of water-soaked lesions in *P. syringae*-host interactions (Keith *et al.*, 2003). The expression of the *algD* gene, which encodes a key

enzyme in the alginate biosynthetic pathway, correlated with the development of water-soaked lesions in collard leaves (Keith *et al.*, 2003).

The genes responsible for alginate biosynthesis and regulation have been under scrutiny for the past several years in the human pathogen *P. aeruginosa* and the plant pathogen *P. syringae*. It is now accepted that the order and arrangement of the alginate biosynthetic genes in these two pathogens are identical (Buell *et al.*, 2003; Penaloza-Vazquez *et al.*, 1997); however, the environmental signals that trigger the production of alginate are different, which can be explained by the different niches they inhabit. The biosynthetic genes for alginate include *algA*, *C*, *D*, *E*, *F*, *G*, *I*, *J*, *K*, *L*, *X, 8* and *44*. With the exception of *algC*, the biosynthetic genes are clustered together in the genomes of both *P. aeruginosa* and *P. syringae*.

Biosynthesis of alginate begins with fructose-6-phosphate that is converted into GDP-mannuronate in four steps that involve the products of *algA*, *algC*, and *algD* (Ramsey & Wozniak, 2005). GDP-mannuronate is then polymerized by Alg8, 44, X, and K. After polymerization, bacterial alginate is acetylated and epimerized by AlgF, I, J and G. Mature alginate is then secreted via an outer membrane porin encoded by *algE*. Alginate biosynthesis is under the control of the *algD* promoter, because *algD* is the first gene transcribed in the gene cluster (Chitnis & Ohman, 1993; Shinabarger *et al.*, 1991). In addition, *algA*, which encodes a bifunctional enzyme that participates in the first and third steps of alginate biosynthesis, is also required for alginate biosynthesis. The *algF*, *algI,* and *algJ* genes, which are responsible for acetylation of the mannuronic residues (Franklin & Ohman, 2002), are not absolutely required for alginate biosynthesis in *P. syringae*; however, the amount of alginate produced when these genes are not expressed is reduced (Penaloza-Vazquez *et al.*, 1997). The AlgG, AlgK, AlgX and Alg44 proteins,

according to the most recent model proposed by Jain and Ohman (Jain & Ohman, 2005), may form a scaffold in the periplasmic space, thus protecting the mature alginate from alginate lyase activity, which is encoded by the *algL* gene. Evidence for this model comes from mutants lacking AlgG, AlgK or AlgX that secrete high levels of low-molecular-weight uronic acids, which are the products of alginate degradation by AlgL (Jain & Ohman, 1998; Robles-Price *et al.*, 2004). Interestingly, the *algL* gene was initially found to be dispensable for alginate production in *P. aeruginosa* (Boyd *et al.*, 1993). Similarly in *P. syringae* pv *syringae*, the *algL* gene was not essential for alginate biosynthesis but was required for optimal alginate production (Penaloza-Vazquez *et al.*, 1997). When the research described in this chapter was underway, two papers were published describing the role of AlgL in *P. aeruginosa*. Albrecht and Schiller (Albrecht & Schiller, 2005) demonstrated that *algL* is required for alginate production in *P. aeruginosa* strain FRD1::pJLS3. In addition, Jain and Ohman (Jain & Ohman, 2005) proposed a new role for alginate lyase during alginate biosynthesis and transport. Jain and Ohman (Jain & Ohman, 2005) suggest that AlgL maybe part of a protein scaffold composed of AlgG, Alg44, AlgK, and AlgX in the periplasmic space, which facilitates transport of alginate to the extracellular milieu. Thus, according to this model AlgL, might be a bifunctional enzyme involved in both the modification of alginate and its transport. However, it is important to note that the role of *algL* in *P. syringae* remains unclear.

P. syringae pv. *tomato* strain DC3000 (*Pst* DC3000) is a pathogen of tomato, *Brassica* spp. (cabbage, cauliflower, collard), and *Arabidopsis thaliana* (Moore *et al.*, 1989; Whalen *et al.*, 1991; Zhao *et al.*, 2000). *Pst* DC3000 has gained importance as a model organism for studying plant-microbe interactions, mainly because of its genetic tractability, its pathogenicity on *A. thaliana* and tomato plants, and the availability of its

genomic sequence (Buell *et al.*, 2003). In this study, the role of alginate lyase in *Pst* DC3000 was investigated. A nonpolar *algL* mutant was constructed in *Pst* DC3000, and the involvement of the *algL* gene in alginate production was investigated. Furthermore, *algL* was shown to be a critical component in the pathogenesis of *Pst* DC3000 on collard plants.

MATERIAL AND METHODS

Bacterial strains, plasmids, and media

The bacterial strains and plasmids used in this study are listed in **Table 3**. *Pst* DC3000, *P. syringae* pv. *syringae* FF5 and derivatives were maintained on mannitol-glutamate (MG) medium (Keane *et al.*, 1970) at 28°C. *E. coli* was routinely maintained on Luria-Bertani (Miller, 1972) medium at 37°C. Rifampicin (25), kanamycin (25), tetracycline (25), chloramphenicol (12.5), and ampicillin (100) (amounts shown indicated in μg/ml) were added to the media when needed. For induction of alginate, *Pst* DC3000, CA1046 and derivatives were grown in Bruegger and Keen (BK) media (Bruegger & Keen, 1979) supplemented with glucose (25g/L) or sodium gluconate (25g/L). The composition of BK per liter is: $(NH_4)_2SO_4$ (1.32 g), $MgSO_4$ (0.08 g), $ZnSO_4$ (0.008 g), potassium phosphate (8.709 g) and casamino acids (2.3 g). For the motility assay King's medium B (King *et al.*, 1954) containing 0.4% agar was used.

Table 3: Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics[1]	Reference or source
E. coli DH5α	$\Delta(lacZYA\text{-}argF)_{u169}$	(Sambrook *et al.*, 1989)
P. syringae		
pv. *tomato* DC3000	Rif^r; alginate producer; pathogenic on tomato, crucifers, and Arabidopsis	(Moore *et al.*, 1989)
CA1046	Rif^r, Km^r; contains Km^r gene in *algL*, derivative of *Pst* DC3000	This study
pv. *syringae* FF5	Cu^r; contains pPSR12, stably mucoid	(Kidambi *et al.*, 1995)
pv. *syringae* FF5.31	Cu^r, Km^r; contains pPSR12, nonmucoid, *algL*::Tn*5*	(Kidambi *et al.*, 1995)
Plasmids		
pUC4K	Km^r; contains 1.3 kb Km^r cassette	Amersham Biosciences
pBBR1MCS	Cm^r; broad-host range cloning vector, replicates well in *Pseudomonas* without selection	(Kovach *et al.*, 1994)
pBluescript SK+	Ap^r; ColE1 origin of replication	Stratagene
pCR2.1	Ap^r, Km^r; 3.9-kb cloning vector; used for cloning PCR products	Invitrogen
pSMC21	Ap^r, Km^r; GFP expression vector	(Kuchma *et al.*, 2005)
pCA1021	Ap^r; contains the *Pst* DC3000 *algL* gene as a 1.1 kb *Kpn*I/*Xba*I fragment in pBluescript SK+	This study
pCA1022	Ap^r, Km^r; contains the *algL* gene in pCA1021 inactivated by the Km^r cassette from pUC4K in pBluescript SK+	This study
pSK2	Tc^r; contains the alginate biosynthetic cluster from *P. syringae* pv. *syringae* FF5 as a 25-kb insert in pRK7813	(Penaloza-Vazquez *et al.*, 1997)
pSK2K74	Tc^r; contains a 7.4 kb *Kpn*I fragment from pSK2, from *algL* to *algA*	(Kidambi and Bender, unpublished results)
pCL	Ap^r, Km^r; contains *algL* from *Pst* DC3000 as a 1.3 kb *Hin*dIII/*Sac*I fragment in pCR2.1	This study
pBL	Cm^r; contains *algL* from *Pst* DC3000 as a 1.3 kb *Hin*dIII/*Sac*I fragment in pBBRMCS1	This study

[1] **Abbreviations: Rif^r (rifampicin), Km^r (kanamycin), Tc^r (tetracycline), Ap^r (ampicillin), and Cm^r (chloramphenicol).**

Molecular genetic techniques

Isolation of plasmid DNA, restriction digests, ligations, PCR, Southern blot and other routine molecular techniques were performed using standard protocols (Sambrook *et al.*, 1989). All enzymes used in genetic manipulations were purchased from New England Biolabs or Invitrogen. DNA fragments were purified from agarose gels using the GeneClean kit (Invitrogen). Nucelotide sequence analysis was provided by the Recombinant DNA/Protein Resource Facility at Oklahoma State University.

Construction of plasmid pCA1022

Dr. Chris Allen, a previous postdoctoral researcher in the Bender lab, constructed an *algL* mutant, but no effort was made to characterize the mutant or publish the results. Her work is summarized here to provide clarity, and she will be included as a co-author on the publication resulting from this thesis chapter. To create an *algL* defective mutant of *Pst* DC3000, Dr. Allen amplified a 1,116 bp fragment containing *algL* from *Pst* DC3000 genomic DNA using primers CA111 (5' G**GGTACC**CCATGCAGACTCCGAAACTGATA) and CA112 (5' GC**TCTAGA**GCCTTGTGGCCTTTTTCATGGG) by PCR. Two restriction sites [(*Kpn*I and *Xba*I, boldface and underlined in CA111 and CA112, respectively)] were added to the sequence during the PCR reaction to facilitate cloning into pBluescript SK+. The PCR product and pBluescript SK+ were then digested with *Kpn*I and *Xba*I, purified from agarose gels, and ligated, resulting in plasmid pCA1021. A kanamycin cassette (1,288 bp) from plasmid pUC4K was used to disrupt *algL*. Briefly, plasmid pUC4K was digested with *Hinc*II (which generates blunt ends), and this fragment was then ligated into the unique *Hinc*II restriction site in pCA1021. The resulting plasmid was named

pCA1022; this was electroporated into *Pst* DC3000 and the resulting *algL* mutant was named CA1046 (see Results).

Complementation experiments

Because of the potential polycistronic organization of the alginate biosynthetic genes in *Pst* DC3000, attempts to complement the *algL* mutant CA1046 were undertaken using the plasmids pSK2, pSK2K74 and pBL. pSK2 contains the entire alginate biosynthetic gene cluster from *P. syringae* pv. *syringae* FF5 (Penaloza-Vazquez *et al.*, 1997), and pSK2K74 contains a 7.4-kb segment of pSK2 spanning *algX* through *algA* (Kidambi and Bender, unpublished results) in pRK415. Plasmid pBL contains the *algL* gene from *Pst* DC3000 in pBBR1MCS (**Figure 5**). Plasmid pRK415 (empty vector) was used as control. For complementation, plasmids pSK2, pSK2K74, and pBL were introduced into CA1046 by electroporation (Sambrook *et al.*, 1989) or conjugation using a triparental mating procedure (Bender *et al.*, 1991), and the successful introduction of each plasmid was confirmed by agarose gel electrophoresis and diagnostic restriction digests.

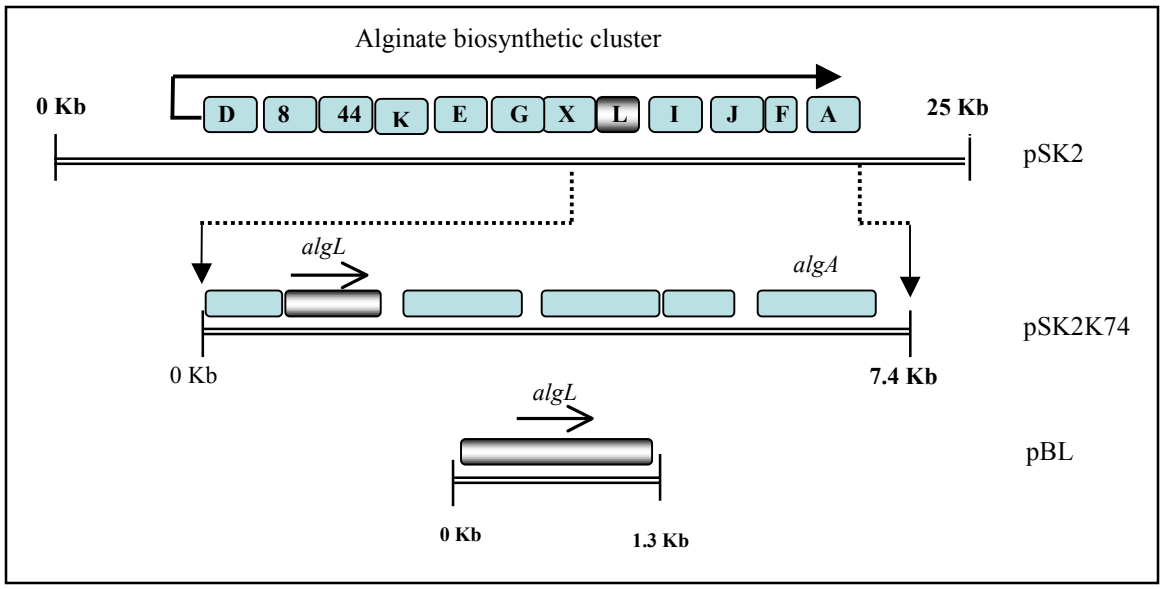

Figure 5: Plasmids used for complementation experiments. pSK2 contains the entire alginate biosynthetic gene cluster from *P. syringae* pv. *syringae* FF5, and pSK2K74 contains a 7.4-kb segment of pSK2 spanning *algX* through *algA*. Plasmid pBL contains the *algL* gene from *Pst* DC3000.

Growth rate

To evaluate if the *algL* mutation impaired growth of CA1046 *in vitro*, *Pst* DC3000, CA1046, and derivatives were grown in BKG and BKGn in 96-well microtiter plates (O'Toole & Kolter, 1998). In brief, *Pst* DC3000, CA1046, and derivatives were grown on Mannitol Glutamate (MG) agar plates for 48 h and used to prepare bacterial suspensions of OD_{600}=0.04. Two hundred microliters of each bacterial suspension were loaded into 10 wells of three different microtiter plates (total of 30 wells for each strain). BKG and BKGn broth were used as blanks. Microtiter plates were incubated on a rotary shaker (200 rpm) at 28°C. The OD_{600} was measured from 0 to 77 h using a microtiter plate reader [Bio-Tek Microplate Reader (www.bio-tek.com)].

Alginate lyase activity in plate assays

Alginate lyase is released to the cellular environment and can be detected in plate assays (Gacesa & Wusterman, 1990; von Riesen, 1980). Initially two different modifications of mannitol glutamate (MG) medium were used to determine alginate lyase activity. One modification contained alginic acid (0.1%) from *Macrocystis pyrifera* (Sigma) (MGAA) with and without 0.3 M NaCl. MGAA medium was solidified with 1% (w/v) Ultra Pure™ agarose (Invitrogen). The presence of alginate lyase activity in this medium was detected by gently flooding plates with a solution of cetyl pyridinium chloride (Sigma) (10% w/v) and incubating for 20 min at room temperature. In this assay, alginate degradation was visualized as a clear zone against an opaque white background (Gacesa & Wusterman, 1990). In the second medium, 0.5% (w/v) activated charcoal (Sigma) was added to MGAA (designated MGAAB) with and without 0.3 M NaCl. In this assay, alginate degradation was visualized as a concave zone surrounding the site of inoculation (von Riesen, 1980).

In other experiments, BK medium (Bruegger & Keen, 1979) supplemented with glucose (25 g/L; BKG) or sodium gluconate (25 g/L; BKGn) was used to assess alginate lyase activity in plate assays.

Isolation and quantification of uronic acid polymers

Alginate is typically extracted from bacterial supernatants as an uronic acid polymer. *Pst* DC3000, CA1046, and derivatives were grown on BKG and BKGn plates (three plates for each strain) at 28°C for 7 days. Each plate was handled separately for quantification of uronic acid polymers, which were isolated using established methods (Jain *et al.*, 2003). Briefly, cells were washed from plates and resuspended in 5 ml of

0.9% NaCl. To precipitate uronic acid polymers, 500 µl of each bacterial suspension was centrifuged at 13,200 rpm for 15 min, and then the supernatant was mixed with 1 ml of 95% ethanol and 50 µl of 3 M sodium acetate. After centrifugation (13,200 rpm), the precipitated alginate was air-dried and resuspended in 250 µl of water. The bacterial pellets were stored at -20°C for total protein quantification. To quantify alginate, 70 µl of the uronic acid polymer suspension was mixed with 600 µl of a solution containing borate-sulfuric acid (100 mM H_3BO_3 in concentrated H_2SO_4) and 20 µl of a carbazole solution (0.1% in ethanol). After mixing, samples were incubated for 30 min at 55°C to facilitate color development. Two hundred microliters of the reaction were loaded into a 96-well microtiter plate and read at 530 nm using a Bio-Tek Microplate Reader. Uronic acid concentrations were determined from a standard plot of alginic acid from *Macrocystis pyrifera* (Sigma).

Total protein from the bacterial pellets was determined using the Bio-Rad (Richmond, CA) protein assay kit as recommended by the manufacturer.

Plant growth and inoculation procedures

Collard (*Brassica oleracea* var. *viridis* L. cv. 'Vates') seedlings were maintained in a growth chamber at 24-25^{o}C, 30-40% relative humidity (RH), with a photoperiod of 12 h. Plants were maintained at 90% RH for 48 h before inoculation. *Pst* DC3000, CA1046 and derivatives were grown for 48 h on MG agar supplemented with antibiotics at 28^{o}C; cells were then suspended to an OD_{600}=0.1 (~10^8 cfu/ml) in sterile distilled water. Silwet L77 (Osi Specialties Inc., Danbury, CT) was added to bacterial inoculum at a concentration of 0.2 µg/ml. Six-week old plants were spray-inoculated with an airbrush

(~8 psi) until leaf surfaces were uniformly wet. After inoculation, collard plants were incubated at 24^{o}C, 60% RH with a 12 h photoperiod for the duration of the experiment.

In the infiltration studies, four-week old tobacco plants (*Nicotiana tabacum*) and six-week old collard plants were infiltrated OD_{600}=0.1 (~10^8 cfu/ml) with *Pst* DC3000, CA1046, and derivatives using established methods (Schaad, 1988). After infiltration, tobacco plants were maintained at 25^{o}C under constant light for 24-48 h, and collard plants were incubated as previously described.

Determination of total and internal bacterial growth *in planta*

Six-week old collard plants were spray-inoculated as described above. Random leaf samples were taken at 0, 1, 3, and 6 days after inoculation. For evaluating the total bacterial population, each leaf was weighed separately (3 replicates per time point) and macerated in 5 ml sterile distilled H_2O. For the internal bacterial population, individual leaf samples (3 replicates per time point) were washed for 5 min in a 15% H_2O_2 solution and twice with sterile distilled water prior to maceration. Bacterial counts were determined by plating dilutions of the leaf homogenate onto MG agar supplemented with antibiotics as needed. Colonies were counted after incubating the plates for 48-96 h at 28^{o}C. Experiments were repeated three times.

Microscopy studies

For microscopy studies, the broad host range GFP expression vector (pSMC21) (Kuchma *et al.*, 2005) was introduced into *Pst* DC3000, CA1046, CA1046(pSK2K74), and CA1046(pBL). Collard, tomato and tobacco detached leaves were infiltrated with bacterial suspensions (OD_{600}= 0.1) using established methods (Schaad, 1988). After

infiltration leaves were allowed to dry, and then individual leaves were maintained in Petri dishes containing 1% water-agar to maintain high humidity. The experiment was also performed on leaves that remained attached to the plant for a three-day period. Infiltrated collard and tobacco plants were incubated as described previously. After two (tobacco) and three (collard) days post infiltration, leaves were inspected with a stereoscope microscope (SZHILLK, Olympus™, Melville, NY) at 30X magnification equipped with EGFP filter cube. Photographs were taken using a Nikon Coolpix model 995 attached to the Olympus™ stereo microscope. All images were compiled using Adobe Photoshop, version 6.0 (Adobe Systems).

RESULTS

Construction of the alginate lyase defective mutant

To create the alginate lyase (AlgL) defective mutant, plasmid pCA1022 (containing *algL*::Km^r) was electroporated into *Pst* DC3000. After electroporation, a potential *algL* mutant named CA1046 was recovered. To confirm the insertion of the kanamycin cassette in *algL*, genomic DNA of *Pst* DC3000 and CA1046 were digested with *Eco*RI, electrophoresed on 0.7% agarose gel at 40 V overnight, transferred to nylon Hybond-N membranes (Amersham Biosciences) and fixed to membranes using UV light. Hybridization probes (*algL* and the kanamycin resistance gene) were obtained from plasmids pCA1021 and pUCK4, respectively, and labeled using the digoxigenin (DIG) DNA Labeling and Detection Kit from Roche Biochemicals (**Figure 6**). The *algL* gene in

Pst DC3000 contains a single *Eco*RI site; thus, *Eco*RI-digested genomic DNA from *Pst* DC3000 results in two fragments (**Figure 6**). If the Km^r gene that does not have an *Eco*RI site is recombined into the genome of CA1046 via a double homologous recombination event, only one of these two fragments will contain the kanamycin cassette. After hybridization with the *algL* probe, two bands were observed in both the *Eco*RI-digested genomic DNA of *Pst* DC3000 and CA1046. However, in *Eco*RI-digested CA1046 DNA, the size of the upper band was consistent with insertion of the 1.3-kb Km^r cassette (**Figure 6**). Moreover, when the Km^r cassette was used as a probe, hybridization occurred in *Eco*RI-digested CA1046 DNA but not to *Eco*RI-digested *Pst* DC3000 DNA, and the hybridizing fragment corresponded to the fragment containing the Km^r-disrupted *algL* gene (**Figure 6**). These results confirmed that the Km^r cassette had inserted into the *algL* gene in strain CA1046.

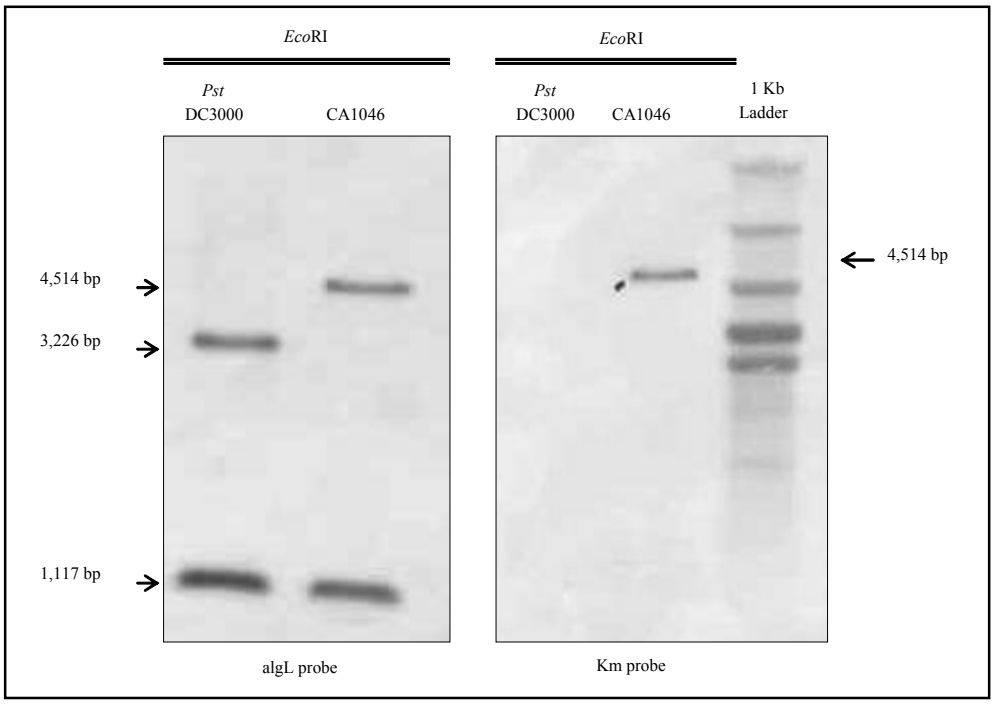

Figure 6: Southern blot analysis of *Eco*RI-digested genomic DNA from *Pst* DC3000 and CA1046. The *algL* gene from pCA1021 and the Km^r cassette from pUCK4 were used as hybridization probes.

To evaluate if the mutation in strain CA1046 impaired its ability to grow *in vitro*, *Pst* DC3000, CA1046 and derivatives were grown in BKG and BKGn broth using a 96-well microtiter plate assay. The results in **Figure 7** indicate that all strains grew equally well when glucose (**Figure 7A**) and gluconate (**Figure 7B**) were supplied as carbon sources. These results indicated that the *algL* mutation had no effect on growth *in vitro*. However, with gluconate, the OD_{600} obtained at 77 h was consistently lower than that obtained with glucose (**Figure 7B**). This difference can be explained by the lower metabolic energy that is obtained from gluconate, compared to glucose.

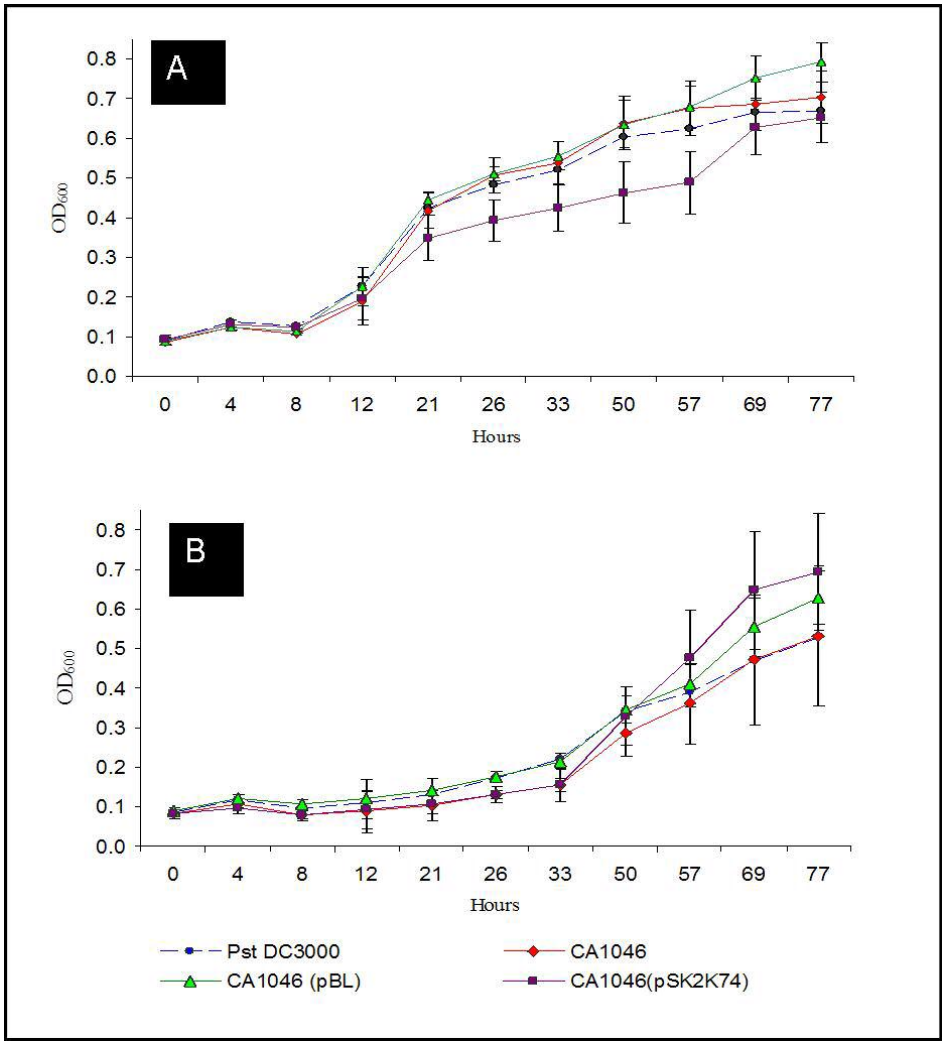

Figure 7: Growth curves of *Pst* DC3000, CA1046, and derivatives in BK broth supplemented with (A) glucose and (B) gluconate incubated at 28°C, 200 rpm.

Alginate lyase activity and complementation experiments

To further characterize the CA1046 strain, *Pst* DC3000 and CA1046 were evaluated for alginate lyase activity using MGAA and MGAAB media. *P. syringae pv. syringae* (*Pss*) FF5(pPSR12) (alginate producer) (Kidambi *et al.*, 1995) and FF5.31 (*algL* mutant) (Penaloza-Vazquez *et al.*, 1997) were included as positive and negative controls, respectively. To prepare MGAA, MG broth was amended with 0.01% (w/v) alginate from *Macrocystis pyrifera* (Sigma) and 0.3 M NaCl and solidified with 1% (w/v) Ultra Pure™ agarose (Invitrogen). To detect alginate lyase activity, MGAA plates were gently flooded with a solution of cetyl pyridinium chloride (CPC, 10% w/v) and incubated for 20 min at room temperature. Alginate degradation was apparent by the formation of clear zones surrounding the site of inoculation (see below). MGAAB was prepared by amending MG broth with 0.1% alginate, 0.3 M NaCl, 1% agar and 0.5% (w/v) activated charcoal (Sigma). The addition of charcoal to this medium facilitates the evaluation of AlgL activity. After inoculation, plates where alginate was degraded were visualized as concave zones surrounding the site of inoculation.

After incubation for 7 days at 28^{o}C, clear zones (**Figure 8A**) appeared after the addition of CPC and concave zones (**Figure 8B**) were observed in MGAA and MGAAB media inoculated with *Pst* DC3000 and FF5(pPSR12), but not in media inoculated with CA1046 or FF5.31. These results not only confirmed that the mutation in CA1046 abolished alginate lyase activity but also established an easy method for future complementation experiments.

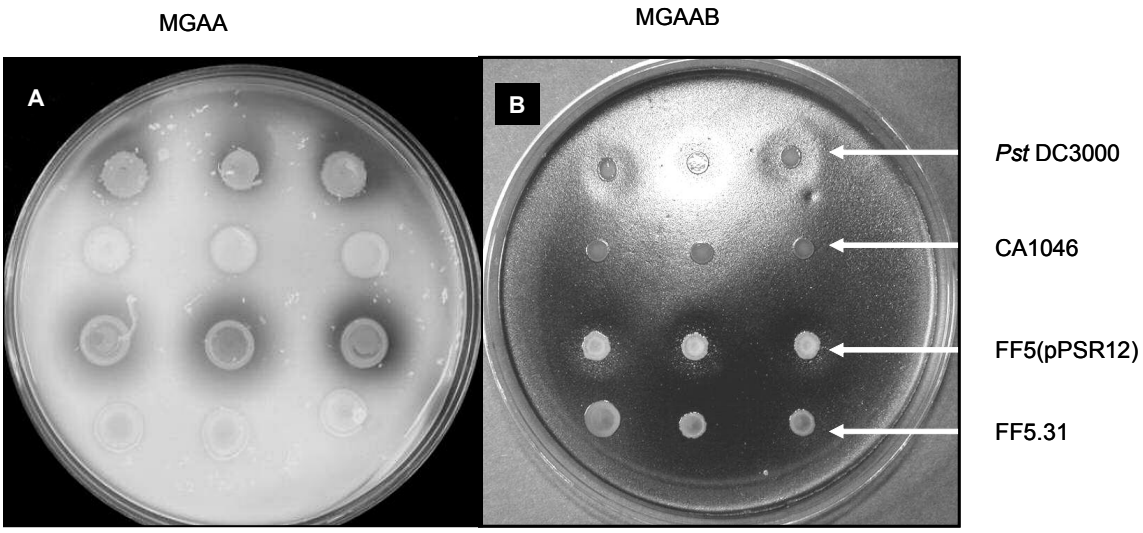

Figure 8: Assay for alginate lyase activity of *Pst* DC3000, CA1046, *Pss* FF5(pPSR12), and FF5.31 after a 7-day incubation in (A) MGAA and (B) MGAAB media. Alginate lyase activity was detected in MGAA (see zones of clearing) after the addition of CPC (panel A). In MGAAB medium, alginate lyase activity was apparent by the formation of concave zones surrounding the point of inoculation (see panel B).

The inactivation of *algL* in the mutant CA1046 may block the expression of downstream genes (*algI, J, F, A*) in the alginate biosynthetic cluster. For this reason, the complementation of CA1046 was attempted using plasmids pSK2, pSK2K74, and pBL. Plasmid pSK2 contains the entire alginate biosynthetic gene cluster (*algD-algA*) in a 25-kb genomic fragment from *Pss* FF5 in pRK7813 (Penaloza-Vazquez *et al.*, 1997), whereas pSK2K74 contains a 7.4-kb segment of pSK2 spanning a portion of *algX* through *algA* (Kidambi and Bender, unpublished results) in pRK415. Plasmid pBL contains the *algL* gene from *Pst* DC3000 in pBBR1MCS. For complementation of CA1046, the successful introduction of pSK2, pSK2K74, and pBL was confirmed by agarose gel electrophoresis and restriction digests (data not shown).

Pst DC3000, CA1046 and CA1046 containing pSK2, pSK2K74, or pBL were evaluated for alginate lyase activity using MGAA and MGAAB as described above. In MGAA and MGAAB media inoculated with *Pst* DC3000, CA1046(pSK2),

CA1046(pSK2K74), and CA1046(pBL) alginate was degraded indicating that alginate lyase activity can be restored with any of the three plasmids. To further confirm these results, the experiment was repeated using the alginate-inducing media, BKG and BKGn, amended with 0.01% alginate, 0.3 M NaCl, and solidified with 1% Ultra Pure™ agarose (as in MGAA) or 0.1% alginate, 0.3 M NaCl, 1% agar and 0.5% (w/v) activated charcoal (as in MGAAB). As in the previous experiment, *Pst* DC3000, CA1046(pSK2), CA1046(pSK2K74) and CA1046(pBL) but not CA1046 degraded alginate (**Table 4** and **Figure 9**).

Table 4: Alginate lyase activity in plate assays.

	Alginate lyase activity on selected media*			
Strains	**MGAA**	**MGAAB**	**BKGAA**	**BKGAAB**
Pst DC3000	+	+	+	+
CA1046	-	-	-	-
CA1046(pSK2)	+	+	+	+
CA1046(pSK2K74)	+	+	+	+
CA1046(pBL)	+	+	+	+

* (+), Alginate lyase activity was visualized either as a clear zone against an opaque white background (MGAA, BKGAA and BKGnAA) or as a concave zones surrounding the site of inoculation (MGAAB, BKGAAB and BKGnAAB). (-) Colonies did not show alginate lyase activity; e.g. no clearing on MGAA, BKGAA or BKGnAA, and no concave zones on MGAAB, BKGAAB and BKGnAAB.

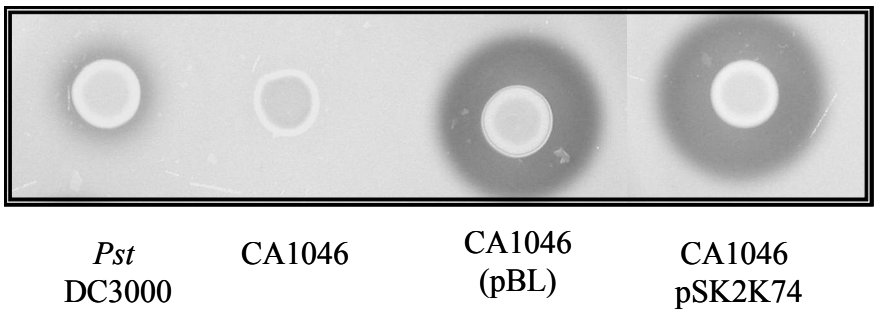

Figure 9*:* Assay for alginate lyase activity on BKGnAA medium*. Pst* DC3000, CA1046, and derivatives were evaluated for alginate lyase activity on BKGnAA medium. After a 7-day incubation period at 28^{o}C, plates were gently flooded with a 10% CPC solution. Alginate lyase activity was characterized as a clear zone against an opaque white background.

Symptom development

The ability of *Pst* DC3000, CA1046, and derivatives of CA1046 to cause disease on collard plants was investigated in spray inoculation experiments. Symptoms were assessed daily for a one-week period following inoculation. Water-soaked lesions surrounded by a chlorotic halo were evident on plants inoculated with *Pst* DC3000, CA1046(pSK2), CA1046(pSK2K74) and CA1046(pBL) beginning three days after inoculation (**Figure 10**). Collard plants inoculated with CA1046 remained symptomless throughout the experiment (**Figure 10**). These results suggest that the *algL* mutation in CA1046 interferes with the ability of the bacterium to cause symptoms. Moreover, this experiment also confirms that the mutant can be complemented for symptom development with the complete alginate biosynthetic cluster (pSK2), with a portion of the cluster (*algX* through *algA*) (pSK2K74), and with *algL* gene alone (pBL).

Total bacterial population on collard plants

The populations of *Pst* DC3000, CA1046, CA1046(pSK2K74), and CA1046(pBL) were evaluated on six-week-old collard plants. The populations of *Pst* DC3000 and CA1046(pBL) increased steadily from 1-3 days after inoculation and were slightly lower at the six-day sampling time (**Figure 11**). In general, the population of CA1046(pSK2K74) was lower than those of *Pst* DC3000 and CA1046(pBL) from 1-3 days after inoculation. At six days, the population of the wild-type and complemented strains was three logs higher that the mutant CA1046 (**Figure 11**). The population of the mutant CA1046 at three days was similar to the population at day zero, suggesting that the mutant is surviving but not multiplying in collard leaves. However, at the sixth day after inoculation, the population of CA1046 was lower than that at day zero, indicating

that the mutant beacame impaired with respect to its ability to survive and multiply in collard (**Figure 11**). These results and the absence of symptoms (**Figure 10**) suggest that the mutation in CA1046 interferes with the ability of the bacterium to establish, multiply and cause symptoms in collard leaves. Furthermore, theses traits were restored when the *algL* gene was present *in trans* in CA1046 (see CA1046(pBL) in **Figure 10** and **11**).

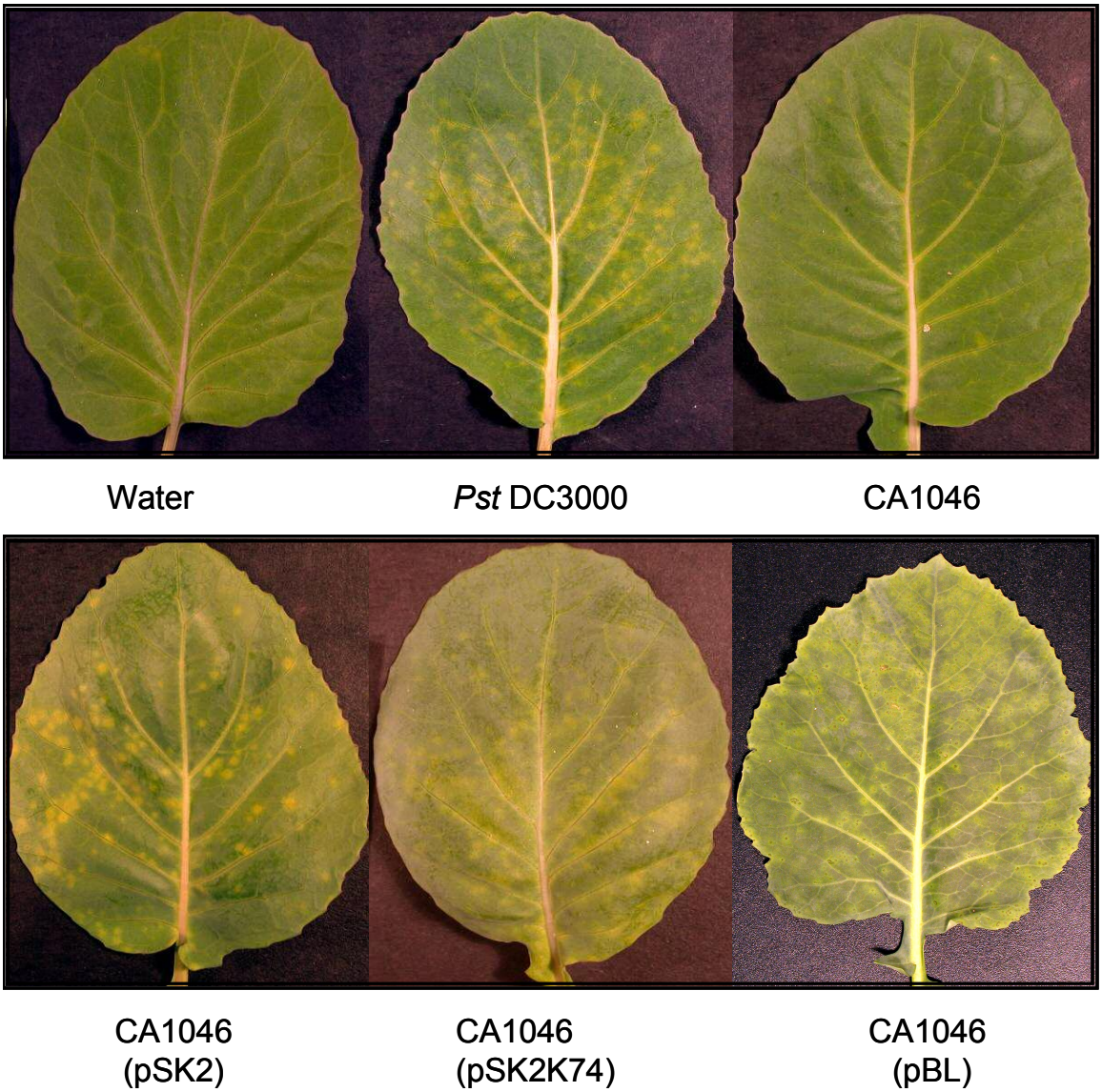

Figure 10: Symptoms on collard leaves spray-inoculated with *Pst* DC3000, CA1046, and derivatives. Bacterial suspensions (OD_{600}= 0.1) were prepared as described in methods. After inoculation, collard plants were incubated at 24^{o}C, 60% RH with a 12 h photoperiod for the duration of the experiment. Photographs were taken 3 days after inoculation.

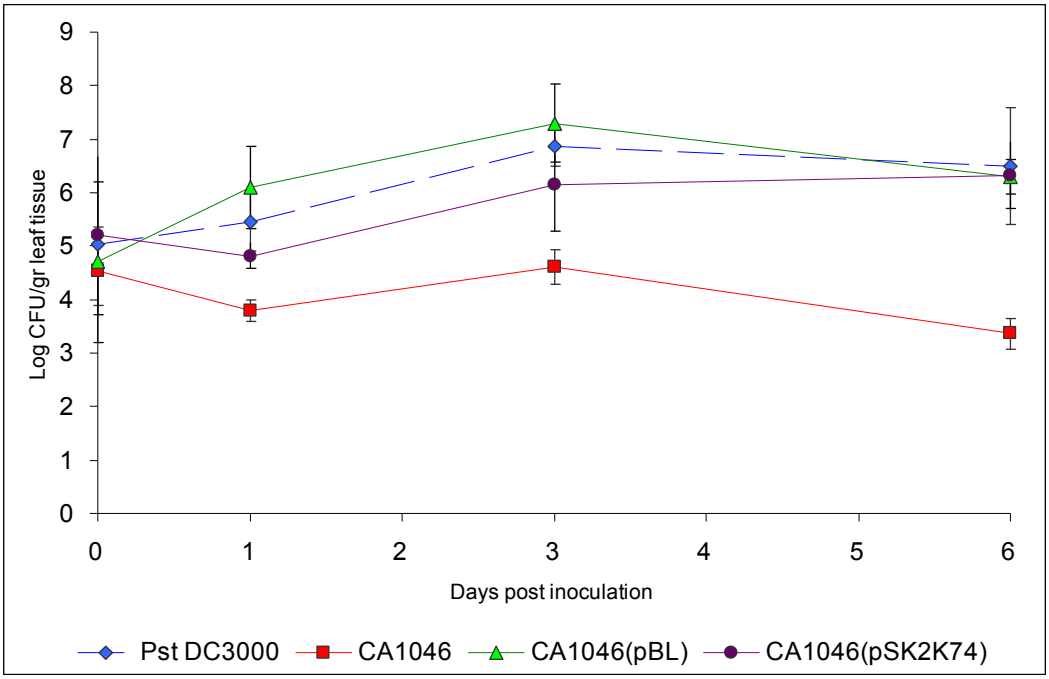

Figure 11: Total bacterial population of *Pst* DC3000, CA1046, CA1046(pSK2K74), and CA1046(pBL) in collard leaves.

Internal bacterial population on collard plants and infiltration studies

The entrance of bacteria into plant tissue is one of the initial steps during the bacterial pathogen-plant host interaction. *P. syringae* enters susceptible host plant tissue through wounds, stomata and other natural openings. A potential impairment in the ability of CA1046 to move or to enter leaf tissue might explain the absence of symptoms on collard plants inoculated with CA1046. To evaluate the former possibility, the motility of *Pst* DC3000, CA1046, and derivatives was evaluated on KMB containing 0.4% agar. After inoculation, plates were incubated at 28^{o}C for 36 h and motility was evaluated visually. No differences among the strains were observed (data not shown), suggesting that the inability of CA1046 to develop symptoms on collard plants is not due to the lack of motility.

Since motility was not impaired in CA1046, the lack of symptoms on collard leaves inoculated with CA1046 might be due to an inability of CA1046 to enter leaf tissue. To assess this possibility, six-week-old collard plants were spray-inoculated with *Pst* DC3000, CA1046, CA1046(pSK2K74) and CA1046(pBL) and the internal populations in collard leaves were evaluated at one, three and six days after inoculation. To preferentially measure the internal population, the external (epiphytic) population was eliminated by surface sterilizing the leaves using a 15% H_2O_2 solution. Leaves were then macerated in sterile distilled water. The population dynamics for the different bacterial strains are shown in **Figure 12**. The bacterial populations immediately after inoculation (day zero) were similar for all strains tested. At day zero, sampled collard leaves were not treated with H_2O_2 solution, hence they represent the total bacterial population applied to plants At the day 1 sampling point, the populations of CA1046(pBL), *Pst* DC3000, CA1046(pSK2K74), and CA1046 were approximately 10^6, 10^5, 10^4, and 10^3 CFU/mg leaf tissue, respectively (**Figure 12**). In general, the internal populations of all strains increased steadily from 1-3 days after inoculation and were slightly lower at the six-day sampling period (**Figure 12**). However, the internal population of CA1046 was significantly lower (1.5-3 logs) than *Pst* DC3000 and CA1046 containing pBL or pSK2K74 from day one to day six. These results suggest that CA1046 is capable of entering collard leaves but perhaps incapable of reaching a level that result in visible disease symptoms on this host.

Infiltration studies

To further investigate if the absence of symptoms on collard plants inoculated with CA1046 was due to a lower internal population as observed in the previous study,

Pst DC3000, CA1046 and derivatives were directly infiltrated into six-week collard detached leaves. Three days after infiltration, strong water-soaked lesion surrounded by a chlorotic halos were observed on the sites infiltrated with *Pst* DC3000, CA1046(pSK2K74), and CA1046(pBL) (**Figure 13**). Interestingly, at three days after infiltration, a dry, small lesion (resembling a HR) was observed at the site inoculated with CA1046; this did not develop into a chlorotic lesion even eight days after infiltration (data not shown). These results suggest that even when a dense bacterial population is infiltrated directly into leaf tissue, the *algL* mutation in CA1046 impairs the ability of this strain to cause symptoms on collard leaves. Similar results were obtained on collard leaves that remained attached to the plant throughout the duration of the experiment (data not shown).

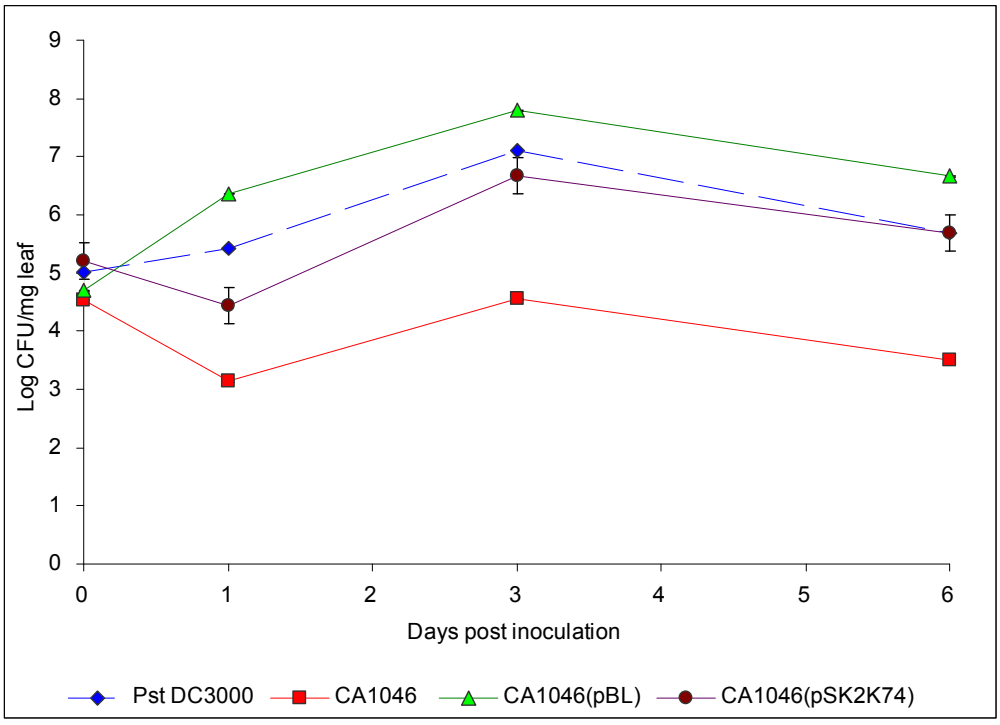

Figure 12: Internal bacterial populations of *Pst* DC3000, CA1046, CA1046(pSK2K74), and CA1046(pBL) in collard leaves.

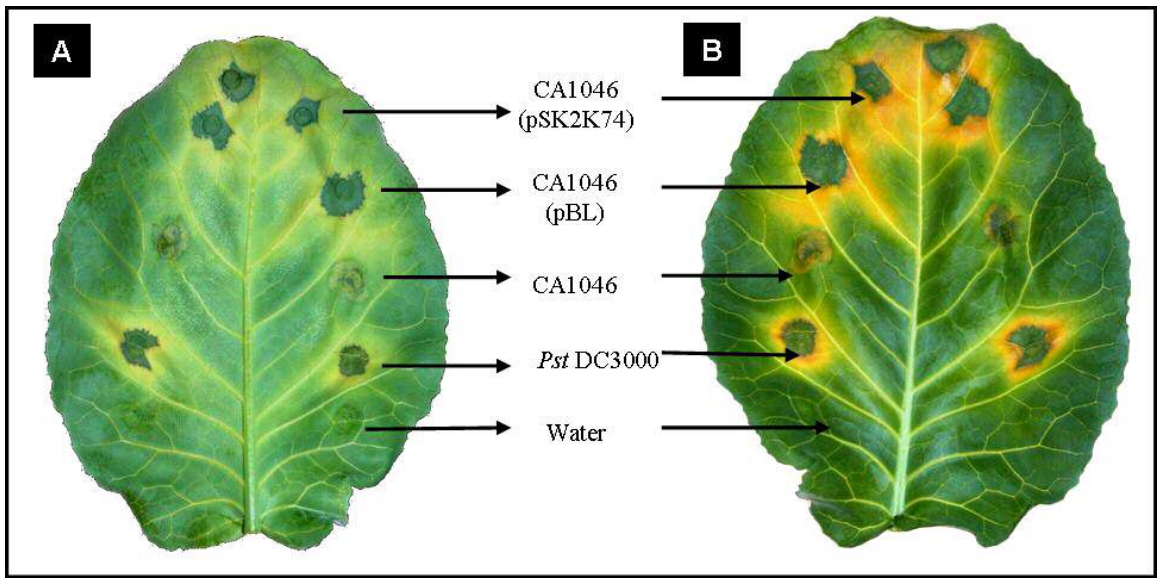

Figure 13: Symptoms on detached collard leaves infiltrated with *Pst* DC3000, CA1046, and derivatives. Bacterial suspensions (OD_{600}= 0.1) were prepared as described in methods. After infiltration, detached leaves were allowed to dry and individual leaves were maintained on 1% water agar to maintain high humidity. Panel A shows symptoms on the underside of the leaf, panel B shows the upper surface of the same leaf. Photographs were taken 3 days after infiltration.

The ability of *Pst* DC3000 to cause disease on host plants is multifactorial, and one of the major factors governing the ability of this bacterium to cause disease is the TTSS. It is well-established that *Pst* DC3000 mutants lacking the TTSS are less virulent on host plants and cannot induce HR on non-host plants. Since the symptoms obtained on collard leaves infiltrated with CA1046 were considerably reduced compared to *Pst* DC3000, the possibility that the mutation in CA1046 impaired the phenotypic expression of the TTSS was evaluated. *Pst* DC3000, CA1046 and derivatives were evaluated for the elicitation of the HR on tobacco plants (**Figure 14**). All strains elicited a typical HR in tobacco leaves when inspected 24 h after infiltration. These results suggest that the mutation of the *algL* gene in CA1046 did not impair the TTSS, and indicate that the reduction in virulence on collard leaves either spray-inoculated or infiltrated with CA1046 can be attributed to the *algL* mutation.

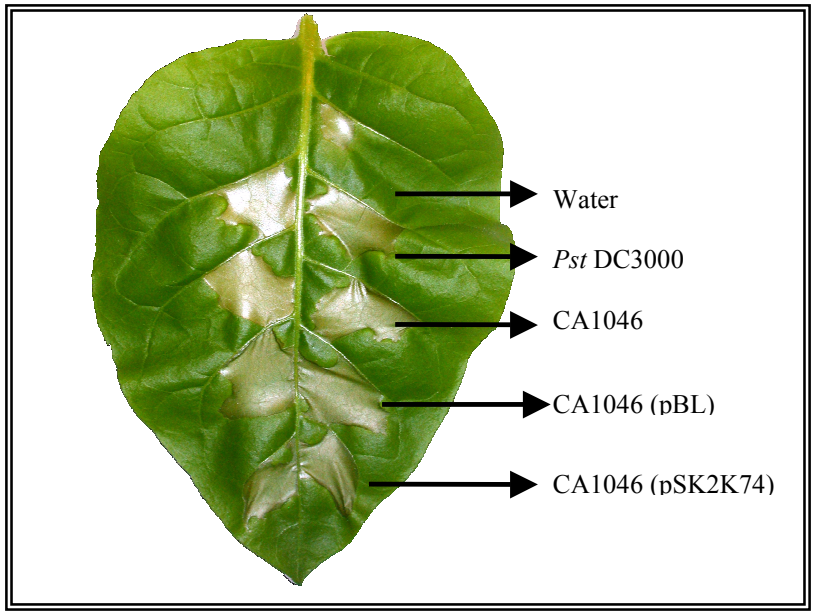

Figure 14: Assay of *Pst* DC3000, CA1046, and derivatives for the hypersensitive response on tobacco leaves. Bacteria (OD_{600}=0.1) were inoculated to tobacco leaves with a needleless syringe. The photograph was taken 48 h after infiltration.

Microscopy studies using green fluorescent protein (GFP)

a) Collard:

The results obtained in population and infiltration studies suggested that CA1046 is able to enter collard leaves but is not able to survive and multiply. A broad host GFP expression vector named pSMC21 was recently used to monitor biofilm formation by *P. aeruginosa* (Kuchma *et al.*, 2005). This plasmid was introduced into *Pst* DC3000, CA1046, CA1046(pSK2K74), and CA1046(pBL) and used to monitor bacterial populations on collard leaves. The bacterial populations were monitored using an epifluorescent stereoscope microscope (Olympus™) equipped with a CGFP filter cube at 30X magnification. Three days after infiltration, strong water-soaked lesions were observed on collard leaves infiltrated with *Pst* DC3000, CA1046(pSK2K74), and CA1046(pBL) as previously described (**Figure 13** and **15**, panels B, D, and E). When

examined under epifluorescent illumination, a strong green fluorescence was observed on collard leaves infiltrated with *Pst* DC3000 and the complemented strains (**Figure 15**, panels B', D', E'). Moreover, the pattern of the fluorescence matched that of the water-soaked lesions. Furthermore, strong GFP expression was observed at the edges of the water-soaked lesions suggesting that bacteria accumulate at the leading edge of the lesions and move toward other sites in the leaf. In contrast, leaves infiltrated with CA1046 did not develop water-soaked lesions (**Figure 15**, panel C), and only few, small green fluorescent areas were detected after epifluorescent illumination (See arrows, **Figure 15,** panel C'). Leaves infiltrated with water were symptomless (**Figure 15**, panel A) and did not show evidence of green fluorescence when examined using epifluorescent illumination (**Figure 15**, panel A'). In summary, the lack of symptoms observed on collard leaves inoculated with CA1046 is due to its inability to survive and multiply inside collard leaves. These results correlate with the reduced bacterial populations of CA1046 (both total and internal) observed in this study.

b) Tomato and tobacco:

Pst DC3000, is a pathogen of tomato in addition to collard; however, the symptoms produced in these two host plants are quite different. Moreover, on tobacco plants, *Pst* DC3000 elicits an HR, which is associated with plant defense (**Figure 14**). Since CA1046 survived poorly inside collard leaves (**Figure 15**, panel C') and no differences were observed with respect to the elicitation of HR on tobacco plants (**Figure 14**), the ability of *Pst* DC3000, CA1046 and derivatives to survive on tomato and tobacco leaves after infiltration was evaluated. Three days after infiltration, tomato leaves infiltrated with all strains showed water-soaked lesions (**Figure 16**, panels B, C, D, and

E). When tomato leaves were examined under UV light, all inoculation sites showed evidence of strong GFP expression (**Figure 16**, panels B', C', D' and E'). These results suggest that CA1046 is able to cause symptoms on tomato leaves and that the *algL* mutation does not impair virulence on tomato plants; these results contrast with those obtained in collard where virulence was greatly reduced (Compare **Figure 15** and **16,** panels C's).

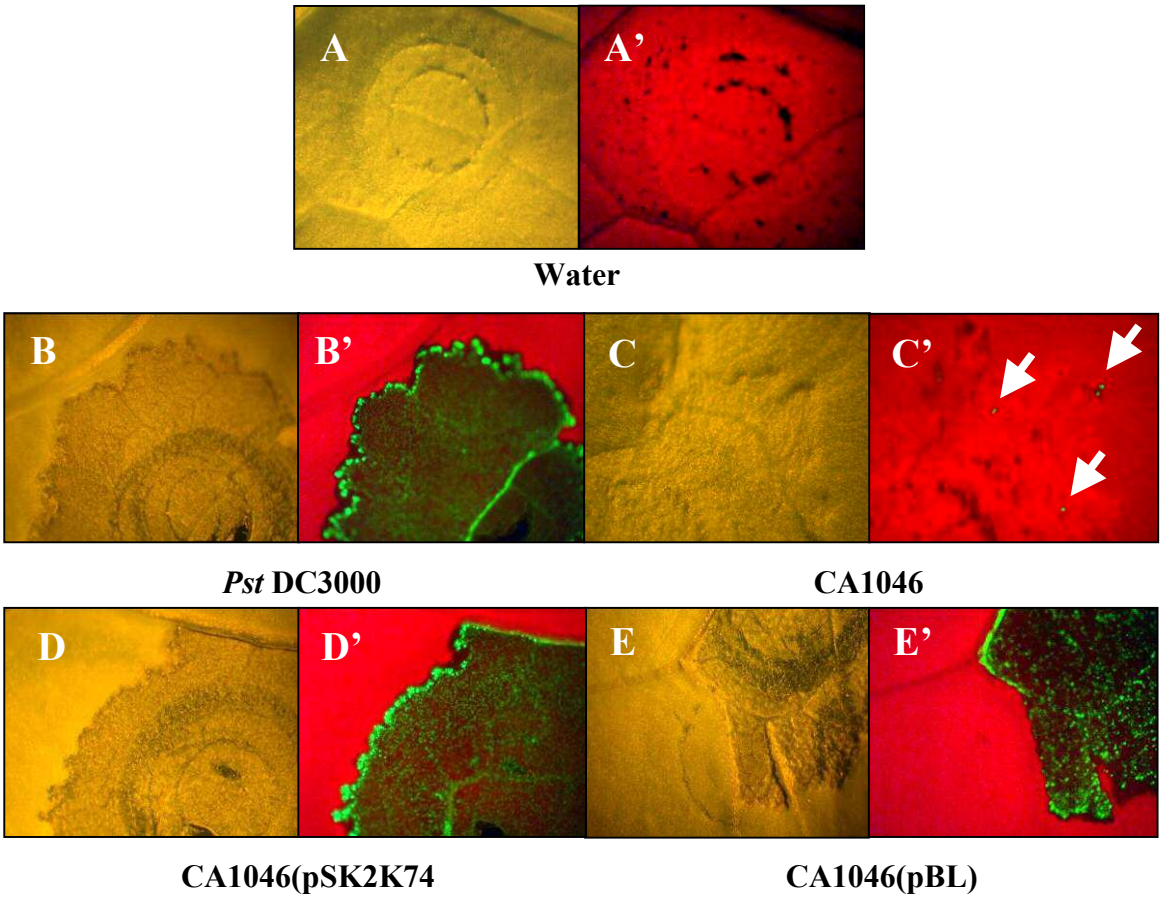

Figure 15: Symptoms on detached collard leaves infiltrated with *Pst* DC3000, CA1046, CA1046(pSK2K74), and CA1046(pBL) carrying the GFP expression plasmid (pSMC21) described by Kuchma (Kuchma *et al.*, 2005). After infiltration, detached leaves were allowed to dry, and individual leaves were maintained on 1% water agar to maintain high humidity. Panels A, B, C, D and E show the site of infiltration at 30X magnification with an epifluorescent stereomicroscope (Olympus ™) three days after infiltration. Panels A', B', C', D', and E' show the same infiltration sites but under epifluorescent illumination. Arrows in panel C' show small, localized areas of green fluorescence in leaves inoculated with CA1046.

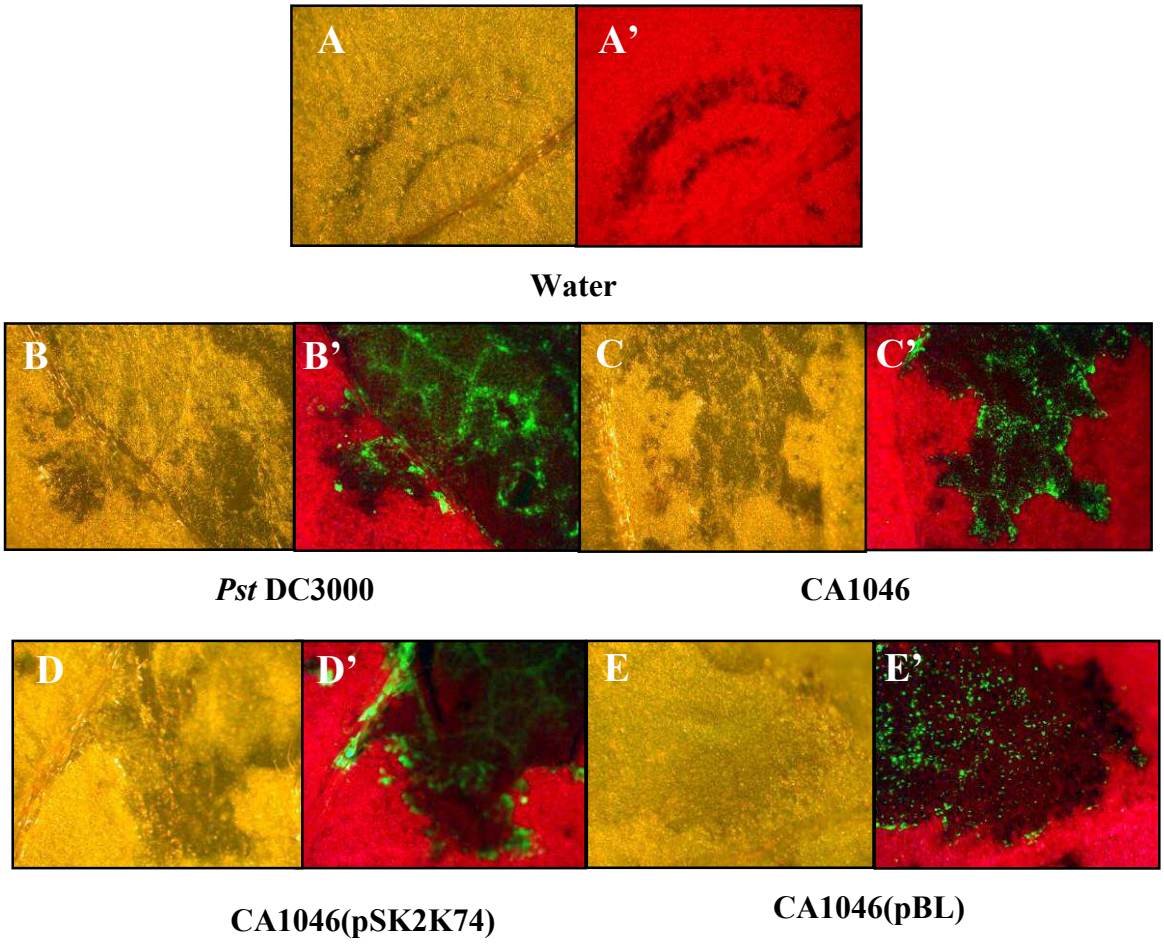

Figure 16: Symptoms on detached tomato leaves infiltrated with *Pst* DC3000, CA1046, CA1046(pSK2K74), and CA1046(pBL) carrying the GFP expression plasmid (pSMC21) described by Kuchma (Kuchma *et al.*, 2005). After infiltration, detached leaves were allowed to dry and individual leaves were maintained on 1% water agar to maintain high humidity. Panels A, B, C, D and E show the site of infiltration examined at 30X magnification using an Olympus ™ epifluorescent stereomicroscope three days after infiltration. Panels A', B', C', D', and E' show the same infiltration sites but under epifluorescent illumination.

On tobacco leaves infiltrated with *Pst* DC3000, CA1046, and derivatives, a typical HR was observed two days after infiltration (**Figure 17**, panels B, C, D, and E). When the areas showing the HR were analyzed under UV light, strong GFP expression was observed in all sites (**Figure 17**, panels B', C', D', and E'). These results suggest that the ability of CA1046 to survive inside tobacco leaves is not impaired when compared to the wild-type and complemented strains.

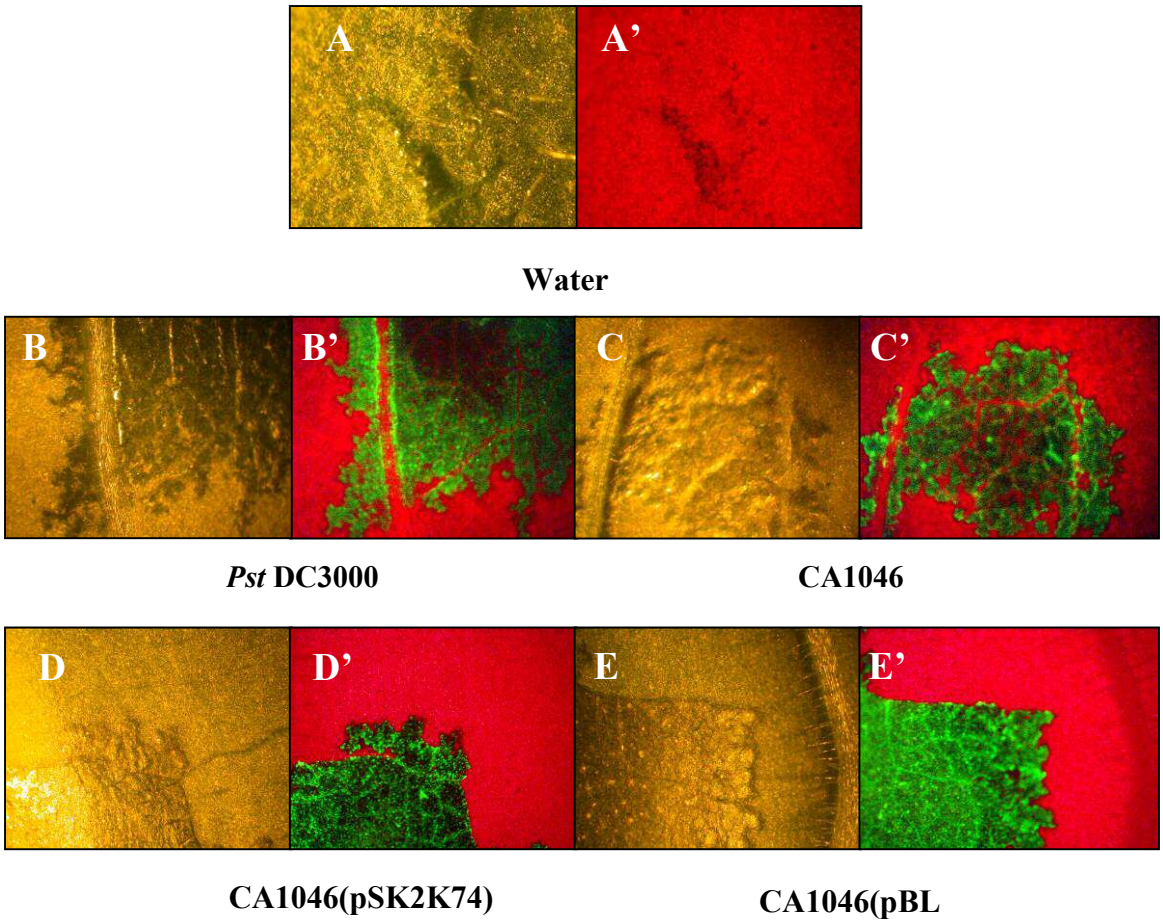

Figure 17: Hypersensitive response on detached tobacco leaves infiltrated with *Pst* DC3000, CA1046, CA1046(pSK2K74), and CA1046(pBL) carrying the GFP expression plasmid (pSMC21) described by Kuchma (Kuchma *et al.*, 2005). After infiltration, detached leaves were allowed to dry and individual leaves were maintained on 1% water agar to maintain high humidity. Panels A, B, C, D and E show the site of infiltration examined at 30X magnification using an Olympus ™ epifluorescent stereomicroscope two days after infiltration. Panels A', B', C', D', and E' show the same infiltration sites but under epifluorescent illumination.

Quantification of uronic acids

The *algL* gene mutated in CA1046 is part of the alginate biosynthetic gene cluster, which is presumably organized into an operon. To evaluate if the mutation in CA1046 interferes with the production of alginate, *Pst* DC3000, CA1046 and derivatives were evaluated for alginate production in Bruegger and Keen (BK) media (Bruegger & Keen, 1979) supplemented with glucose (BKG) or gluconate (BKG). When glucose was used as carbon source, *Pst* DC3000, CA1046, and CA1046(pSK2K74) produced

comparable quantities of alginate. Interestingly, the complemented strain CA1046(pBL) produced more alginate than *Pst* DC3000 and CA1046 but was not significantly different from CA1046(pSK2K74) (**Figure 18**). With the addition of gluconate, CA1046(pBL) and CA1046(pSK2K74) produced alginate at levels comparable to the wild-type strain *Pst* DC3000. Surprisingly, when gluconate was added, the *algL* mutant (CA1046) produced significantly more alginate than any other strain evaluated. The results obtained with glucose and gluconate as carbon sources clearly demonstrated that the amount of alginate produced depends on the carbon source used in the media. In summary, the results convincingly demonstrate that the *algL* mutation in CA1046 did not affect the ability of this strain to produce alginate *in vitro*.

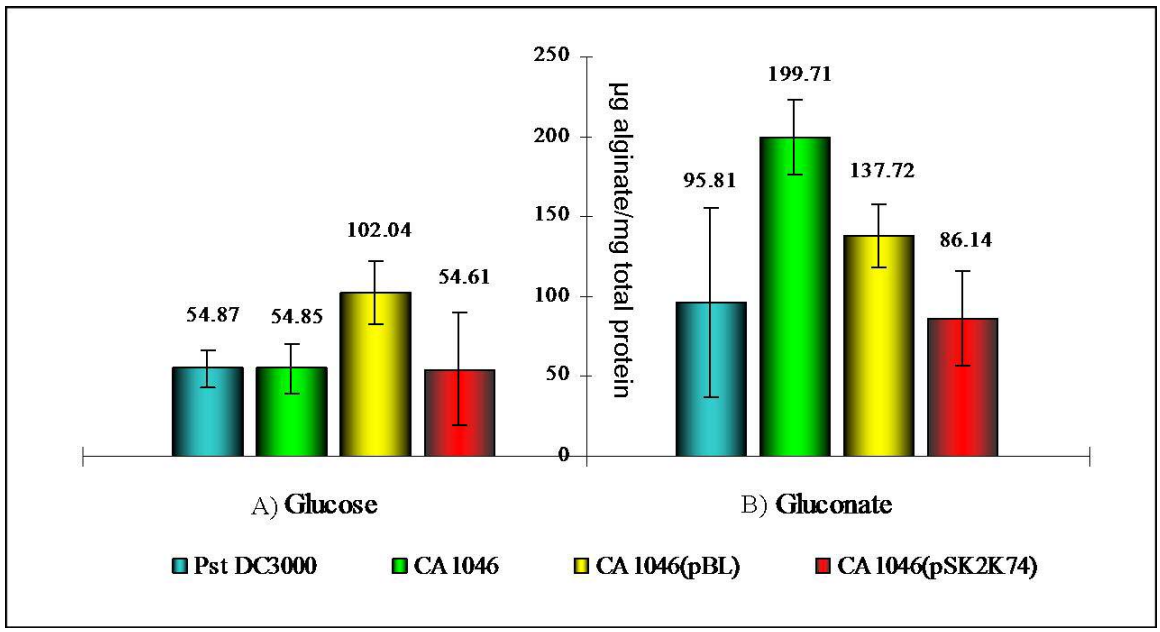

Figure 18: Alginate production by *Pst* DC3000, CA1046, CA1046(pSK2K74), and CA1046(pBL) on BK media supplemented with A) glucose and B) gluconate. The results represent the mean of a minimum of three and a maximum of five independent assays. Each experiment consisted of three plates that were handled separately to quantify alginate as described in methods.

DISCUSSION

Pst DC3000 is a pathogen of tomato, *Brassica* spp. (cabbage, cauliflower, collard), and *Arabidopsis thaliana* (Moore *et al.*, 1989; Whalen *et al.*, 1991; Zhao *et al.*, 2000). Moreover, *Pst* DC3000 is a model organism for studying plant-microbe interactions. The pathogenicity of *Pst* DC3000 depends on various factors, and one factor that was previously implicated in virulence on collard plants was alginate (Keith *et al.*, 2003). However, mutants defective in alginate or alginate-modifying enzymes have not been previously generated for *Pst* DC3000. In this study, an *algL* mutant was constructed in *Pst* DC3000, and the involvement of the *algL* gene in alginate production and in virulence was investigated.

The *algL* gene in *Pst* DC3000 encodes alginate lyase, an enzyme that degrades alginate. To successfully determine whether CA1046 was defective in alginate lyase activity, two assays were used in this study (Gacesa & Wusterman, 1990; von Riesen, 1980). The use of these media clearly demonstrated that alginate lyase activity was abolished in the mutant CA1046 (**Figure 9** and **Table 4**). Furthermore, these assays were shown to be invaluable in determining whether alginate lyase activity was restored in complementation studies.

The alginate biosynthetic genes in *Pst* DC3000 include *algD-8-44-K-E-G-X-L-I-J-F-A* and *algC*. With the exception of *algC*, the biosynthetic cluster is under the control of the *algD* promoter. Since the inactivation of *algL* in the mutant CA1046 could have

disrupted the expression of downstream genes (*algI*, *J*, *F*, *A*) in the alginate biosynthetic cluster, the complementation of CA1046 was assessed using plasmids pSK2, pSK2K74, and pBL. Plasmid pSK2 contains the entire alginate biosynthetic gene cluster (*algD-algA*) on a 25-kb genomic fragment from *Pss* FF5 (Penaloza-Vazquez *et al.*, 1997) whereas pSK2K74 contains a 7.4-kb segment of pSK2 spanning a portion of *algX* through *algA* (Kidambi and Bender, unpublished results). Plasmid pBL contains the *algL* gene from *Pst* DC3000. Derivatives of CA1046 named CA1046(pSK2), CA1046(pSK2K74), and CA1046(pBL) harboring individual plasmid constructs were evaluated for alginate lyase activity on plates. The results obtained showed that alginate lyase activity can be restored using any of these plasmids.

In preliminary experiments, *Pst* DC3000 and CA1046 were evaluated for virulence on collard plants. Interestingly, after a three-day incubation period, plant spray-inoculated with CA1046 did not develop symptoms; these results contrast with the wild-type strain *Pst* DC3000, which developed water-soaked, chlorotic lesions (**Figure 10**). Symptoms were restored in plants inoculated with CA1046(pSK2), CA1046(pSK2K74), and CA1046(pBL), which demonstrates that any of the plasmids used for restoration of alginate lyase activity can also be used for restoration of symptoms. Furthermore, these results suggest that the absence of symptoms in collard plants inoculated with CA1046 can be attributed solely to the *algL* mutation.

The total and internal population dynamics of *Pst* DC3000, CA1046, CA1046(pSK2K74), and CA1046(pBL) suggested that the lack of symptoms in collard plants inoculated with CA1046 was due to its inability to survive and multiply inside collard leaves. Moreover, in collard leaves infiltrated with CA1046, symptoms were

greatly reduced in comparison to *Pst* DC3000 even when high concentrations of bacteria were infiltrated. These findings support the theory that CA1046 is unable to survive inside collard leaves, possibly because events that occur during the pathogen-host interaction (e.g. production of toxic compounds) may prevent bacterial multiplication. Studies with collard leaves infiltrated with *Pst* DC3000, CA1046, CA1046(pBL) and CA1046(pSK2K74) harboring a GFP expression vector further confirm that CA1046 survives poorly inside collard leaves (See **Figure 15**, panel C'). The GFP infiltration studies in collard also demonstrated that *Pst* DC3000 and the complemented strains accumulate at the leading edges of the bacterial lesions. It is well-established that *Pst* DC3000 mutants lacking the TTSS are less virulent on host plants and cannot induce the HR on non-host plants. In this study, infiltration experiments with *Pst* DC3000, CA1046 and derivatives on tobacco plants demonstrated that the phenotypic expression of the TTSS was not abolished in CA1046, thus a nonfunctional TTSS did not cause the lack of symptoms in collard plants inoculated with this mutant. In addition, when *Pst* DC3000, CA1046, and derivatives were analyzed for growth *in vitro*, no differences were observed, suggesting that the *algL* mutation in CA1046 did not impair the ability of the bacterium to grow *in vitro*.

The *algD* gene is the first gene in the biosynthetic cluster to be transcribed and represents the committed step for the synthesis of alginate. In collard plants inoculated with *Pst* DC3000, there was a strong expression of the *algD* gene in water-soaked lesions, suggesting that alginate is important during infection of collard (Keith *et al.*, 2003). In addition, the *algD* gene was differentially expressed in host plants (e.g. high expression in collard and lower expression in tomato) and non-host (tobacco) plants

(Keith *et al.*, 2003). Previous results suggested that alginate may be produced in tomato and tobacco leaves, but the quantity is lower than in collard (Keith *et al.*, 2003). Moreover, on tomato leaves, *Pst* DC3000 induces necrotic lesions surrounded by chlorotic halos without a water-soaked phenotype. When *Pst* DC3000, CA1046, and derivatives harboring the GFP expression plasmid were infiltrated into tomato and tobacco leaves, CA1046 was able to survive as well as the wild-type *Pst* DC3000. In summary, the results obtained in this study support those obtained previously (Keith *et al.*, 2003) and suggest that *algL*, like *algD*, may be more important for pathogenesis in collard plants and less important in tomato plants.

The high expression of the *algD* gene in collard leaves (Keith *et al.*, 2003) correlates with the appearance of water-soaked lesions, which may be the result of alginate production. In *P. syringae* pv. *syringae,* an *algL* mutant was still able to cause symptoms on the host plants but alginate was not produced due to the inactivation of the downstream genes (*algI*, *J*, *F*, *A*) (Yu *et al.*, 1999). Moreover, alginate production was partially restored when the *algA* gene was expressed *in trans*, suggesting that the *algL-I-J-F* genes are not essential for alginate production at least *in vitro*. The production of alginate *in vitro* by several pseudomonads has been previously demonstrated to be largely dependent on the media and growth conditions (Chan *et al.*, 1984; Fett & Dunn, 1989; Fett *et al.*, 1989; Kidambi *et al.*, 1995; Osman *et al.*, 1986). In this study, several media were evaluated (data not shown) and a medium that facilitated optimal production of alginate by *Pst* DC3000 was identified. The results obtained with this medium (**Figure 18**) clearly show that alginate production was not abolished in the *algL* mutant, CA1046. Thus, the *algL* gene may not be essential for alginate production in *Pst* DC3000 *in vitro*,

and these results agree with those observed for other *P. syringae* pathovars (Penaloza-Vazquez *et al.*, 1997; Yu *et al.*, 1999). In *P. aeruginosa*, it was originally suggested that the *algL* gene was not important for alginate production (Boyd *et al.*, 1993). However, in a recent study, a *P. aeruginosa* strain was created by artificially replacing the promoter of the *algD* operon with a $LacI^q$-repressed *tac* promoter, which permits IPTG induction of the operon. In this genetic background, the non-polar mutation of the *algL* gene disrupted alginate production (Albrecht & Schiller, 2005), and induction of the *algD* operon by IPTG was lethal (Jain & Ohman, 2005). The authors suggested that the *algL* mutant still produced alginate after IPTG induction; however, instead of being exported to the extracellular milieu, alginate accumulated inside the cells and the bacteria died, which suggests a role for alginate lyase in the export of alginate. If this model is true, it may explain why CA1046 is unable to survive inside collard leaves where there is a high expression of the *algD* gene and hence high production of alginate (Keith *et al.*, 2003). Moreover, in tomato and tobacco leaves, *algD* is expressed at lower levels compared to collard and hence less alginate would be produced, thus explaining the survival of CA1046 in these two plants. Even though alginate was produced by CA1046 *in vitro*, further experiments are needed to explain differential survival of this mutant *in planta* (discussed below).

In conclusion, even though *algL* mutations have been generated in *P. syringae* pv. *syringae* (Penaloza-Vazquez *et al.*, 1997; Yu *et al.*, 1999), they are polar mutations that result in an alginate-defective phenotype; hence the results are mainly due to lack of alginate production rather than the mutated gene. This is one of the first studies to critically assess the role of an *algL* non-polar mutation *in planta* and the first alginate

biosynthetic gene mutated in *Pst* DC3000. The results obtained with mutant CA1046 in this study clearly demonstrate that the mutation in the *algL* gene has a profound effect in virulence on collard leaves but not in tomato leaves. Future studies will use an *algD* mutant of *Pst* DC3000 to further investigate whether the difference in the inability of CA1046 to survive in collard and tomato leaves is because of alginate or because of the *algL* mutation.

CHAPTER IV

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VITA

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Candidate for the Degree of

Master of Science

Thesis: ROLE OF ALGL (ALGINATE LYASE) IN THE VIRULENCE OF P*SEUDOMONAS SYRINGAE* PV *TOMATO* DC3000

Major Field: Plant Pathology

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Title of Study: ROLE OF ALGINATE LYASE IN THE VIRULENCE OF *PSEUDOMONAS SYRINGAE* PV *TOMATO* DC3000

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Scope and Method of Study: The purpose of this study was to evaluate the role that alginate lyase plays during pathogen-host interaction in *P. syringae*. An *algL* mutant was constructed in *Pst* DC3000 by inserting a kanamycin resistant cassette within the *algL* sequence. The involvement of the *algL* gene in alginate production and in virulence of collard plants was investigated. This study will enhance our understanding of alginate and alginate lyase in plant pathogen interactions.

Findings and Conclusions: This is one of the first studies to critically assess the role of an *algL* non-polar mutation *in planta* and the first alginate biosynthetic gene mutated in *Pst* DC3000. In collard plants the bacterial population was lower compared to the wild-type strain *Pst* DC3000. Suggesting that CA1046 is not able to survive and multiple in collard leaves. In contrast, in tomato leaves, CA1046 cause similar symptoms as the wild-type. In summary, the results obtained with mutant CA1046 in this study clearly demonstrate that the mutation in the *algL* gene has a profound effect in virulence on collard leaves but not in tomato leaves. Moreover, the *algL* mutation in CA1046 did not prevent alginate production *in vitro*, suggesting that *algL* is not required for alginate biosynthesis in *Pst* DC3000.

ADVISER'S APPROVAL: Carol L. Bender